CONDITIONED EFFECTS OF HEROIN ON PROINFLAMMATORY MEDIATORS

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ABSTRACT

JENNIFER LYNN SZCZYTKOWSKI: Conditioned Effects of Heroin on Proinflammatory Mediators
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Heroin administration alters the induction of nitric oxide, a molecule known to play a critical role in immune function, as well as the production of several proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-one beta (IL-1β). Previous research has shown that these alterations can be conditioned to environmental stimuli that have been associated with drug administration. The investigations presented here deal with the neural mechanisms whereby these drug cues alter the expression and production of certain proinflammatory mediators essential for host defense. The experiments in Chapter 2 demonstrate that the conditioned alterations in proinflammatory mediators induced by exposure to previously heroin-paired stimuli are a true form of associative learning as they are susceptible to both extinction and latent inhibition. Chapter 3 reveals that the basolateral amygdala (BLA), an area of the brain which has been implicated in the formation of stimulus-reward associations within models of drug abuse, is necessary for the expression of the conditioned response. Administration of a combination of the GABA agonists, muscimol and baclofen, directly into the BLA blocked the conditioned suppression of proinflammatory mediators while administration of these same drugs into an adjacent region of the caudate did not alter the response. The experiments outlined in Chapter 4 show that the pharmacological blockade dopamine,

D_1 receptors in the BLA reverses the conditioned suppression induced by exposure to previously heroin-paired stimuli. The administration of the dopamine D_1 antagonist, SCH23390, but not the D_2 antagonist, raclopride, resulted in attenuation of the conditioned effect. These studies are important because they are the first to investigate the neural circuitry involved in the conditioned immune alterations produced by exposure to drug cues. Overall, these findings indicate a need to consider the implications of exposure to drug cues on the immune functioning of current and recovering heroin use.

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CHAPTER 1

GENERAL INTRODUCTION

A Brief History of Heroin & Heroin Use

Heroin, or diacetylmorphine, is a semisynthetic derivative of morphine and a member of the class of drugs known as opiates. Opiates include any of various sedative narcotics found in opium or any natural or synthetic derivatives thereof. Opium is found in the dried sap of the opium poppy and naturally contains over two dozen alkaloids, including morphine and codeine. The opium poppy, *Papaver somniferum*, is one of over 250 species of poppy plants and derives its name from the latin word for 'sleep inducing'. It is unknown whether the *P. somniferum* plant naturally evolved from another species of poppy, such as the *Papaver setigerum* which contains minute amounts of opium, or whether it was specifically cultivated and bred by humans to maximize the plant's narcotic effects. It is also a mystery as to why the plant would produce the alkaloid substance at all. Theories suggest that the opium may aid in the production of the poppy plants' seeds or that it protects the plant from unwanted predators. Perhaps the most intriguing theory of all claims that the opium poppy produces the addictive medicinal substance to ensure its continued human cultivation (Booth, 1996).

Some of the first recorded evidence of opium cultivation comes from the history of the Sumerian culture. The Sumerians are credited with developing the first form of writing around 3300 BC and it is from their writings that we first learn of the

opium poppy and its use both medicinally and recreationally. The Sumerians referred to the plant as *hul gil* or 'plant of joy' and as time passed the secrets of opium cultivation were shared with neighboring civilizations thus allowing for its introduction into cultures such as those of the Syrians, Egyptians and Greeks. Although the Sumerians were the among the first to record their interaction with the opium poppy it is possible that the origins of its use reach much further back into history. Cultivated seeds from the *P. Somniferum* poppy were among the items discovered at the site of a 4500 year old Cortaillod village in Switzerland though we know little of how the plant was used by the villagers. The oldest known physical evidence of opium cultivation was found at the 7700 year old site of La Marmotta in Italy. Here archaelogists uncovered *P. Somniferum* seeds alongside items believed to be used for religious purposes suggesting the plant might have been a component of spiritual life for these Neolithic farmers (Merlin, 2003).

While medicinal opium use was already widespread by the second millenium, Mithridates VI of Pontus (134-63 BC) is credited with concocting one of the first known opium containing medicines which was aptly named 'mithridate'. Mithridate contained over forty ingredients including opium, saffron, myrrh, castor, ginger and cinnamon and was hailed as an omnipotent antidote for everything from snakebites to poisoning. In fact, according to legend Mithridates VI regularly ingested his cure-all to guard against assassination attempts. The composition of mithridate was later altered by Andromachus, physician to emperor Nero (37-68 AD), and the new formula became known as theriac. For hundreds of years opium was treated almost as a panacea and was prescribed by doctors to treat ailments ranging from kidney stones to morning sickeness to diarrhea. Even the famous Greek physician Galen (129-200 AD) prescribed mithridate to his patients "claiming it resisted poison and venomous

bites and cured, amongst other things, headaches, vertigo, deafness, epilepsy, apoplexy, poor sight, bronchitis asthma, coughs, the spitting of blood, colic, jaundice, hardness of the spleen, kidney stones, urinary complaints, fever, dropsy, leprosy, menstrual problems, melancholy and all other pestilences"(Booth, 1996). Both theriac and mithridate were cited in the London Pharmacopoeia published in 1618 but by the 1756 publishing of the Edinburgh Pharmacopoeia had fallen from favor and were left out.

A more modern opium concoction can be traced to Thomas Sydenham, a physician of the seventeeth century who used a combination of alcohol and opium to treat disease. The term 'laudanum', which is translated *worthy of praise* in Latin, is thought to have been orignally coined by the sixteenth century physician, Paracelsus. However, it is unknown whether Paracelsus' version of laudanum contained opium as an ingredient as his formula remains a mystery (Hodgson, 2001). The tendency to prescribe opium for even the most minor health complaint led to an increasing number of problems concerning its overuse. In 1700, Dr. John Jones published „Mysteries of Opium Reveal'd" in which he wrote about the addictive potential of opium stating that discontinuation of its use resulted in „great, and even intolerable Distresses, Anxieties and Depressions of Spirits, which in a few days commonly end in a most miserable Death". Some physicians went as far as to caution that the drug was in fact poisonous but despite these warnings opium use continued and opium importation into the United States doubled between the years of 1870 and 1890 (Courtwright, 2001).

The physiological effects of opium on the body are the result of its principal active ingredient, morphine. These effects include analgesia, sedation, respiratory depression and constipation. Morphine was originally isolated from the opium poppy

by the German pharmacist Friedrich Wilhelm Serturner in 1805. His discovery was historical not only because of the enormous impact that morphine would have in years to come but also because this was the first alkaloid to ever have been isolated from a plant. He named the isolated substance 'morphium' in reference to Morpheus, the Greek god of dreams because of its sleep-inducing properties. Morphine began to produced commercially between 1820 and 1830, however, the drug was underutilized because ingestion did not have the same magnitude of effects as injection directly into the bloodstream. When Dr. Alexander Wood invented the hypodermic needle in 1853 morphine use became even more popular. In fact, during the Civil War morphine syringes became a standard tool in army field hospitals for treating soldiers' pain. Unfortunately, the rampant use of the drug also led to the development of huge numbers of soldiers turned morphine addicts (Carnwath & Smith, 2002). In fact, it is believe that most of the morphine addicts of this time period had their first experience with the drug for medicinal purposes and continued its use long after the initial medical reasons warranted (Hodgson, 2001).

As morphine addiction began to become a rampant societal issue the quest began for a non-addictive alternative. While working on this very problem in 1874, CR Alder Wright boiled morphine with acetic anhydride effectively altering its chemical structure (Wright, 1874) and creating a narcotic even more powerful and addictive. Thus begins the history of heroin. Following its initial synthesis, heroin was not met with much enthusiasm. Wright invited London physician FM Pierce to determine the effects of this compound and others but after testing in both dogs and rabbits the data was found to be inconclusive (Pierce, 1874). The chemical was tested again in 1888 by David Dott and Ralph Stockman at the University of Edinburgh. The pair reported to the British Medical Association their observed comparisons

between morphine and the new diacetylmorphine compound. In their report, Stockman and Dott claimed that the acetylated morphine compound was more effective than pure morphine at depressing the respiratory centers of the test subjects and that high doses may result in convulsions more frequently than morphine. They seemed relatively uninterested in any possible therapeutic or medicinal properties of diacetylmorphine on its own (Sneader, 1998).

It wasn't until 1897, when Felix Hoffman and Aurthur Eichengrun set upon examining the compound again that its unique medicinal properties began to be explored. Hoffman and Eichengrun were members of a research team set up by Heinrich Dreser at Bayer Pharmaceuticals to investigate the acetylation of various medications. Dreser believed that the process of acetylation would lead to increased potency and reduced side effects of these medications. This same team is also responsible for the acetylation of salicylic acid which resulted in the production of what we know today as aspirin. In a series of papers, Dreser heralded the newly discovered properties of the compound claiming that the acetylated morphine would not only suppress coughing but also had the power to stimulate respiration thus leading to increased clearing of the lungs (Carnwath & Smith, 2002). On the basis of Dreser's reports, Bayer Pharmaceutical Company introduced heroin to the medical community as a cough suppressant and treatment for tuberculosis in 1898. However, Dreser's theory on the stimulatory action of heroin on the respiratory system was soon laid to rest. According to the British Pharmaceutical Codex (1907), the 'introduction of acid (or alkyl) groups into the morphine molecule, however, weakens, though it does not remove, its depressing action on the respiratory centre, and lessens its narcotic effect'. In 1911, von Issekutz published results further proving that heroin acts as a respiratory depressant rather than a stimulant. Furthermore, the 1911 British

Pharmaceutical Codex goes on to state that 'acetomorphine [heroin] resembles codeine, over which it is very doubtful if it possesses any advantage'.

Prior to 1906, the addition of heroin and other opiates in medicinal remedies was mostly unregulated. The twentieth century ushered in a new mentality concerning the unregulated sale and use of drugs and legislation limiting and soon eliminating these practices began to be passed in the United States. In 1906 the Pure Food and Drug Act is enacted requiring medicines to list their ingredients. One regulation that may have actually lended to the increasing number of heroin users was the Smoking Opium Exlusion Act of 1909 which forbade the importation of opium except by pharmaceutical companies. Since opium was now only available illicitly, and thus expensive, many former opium users switch to heroin to feed their addiction because heroin was "cheaper, quicker, and much harder to detect" (Musto, 2002). In 1914, the Harrison Narcotic Tax Act was passed to both control and tax the importation, production and sale of opiates and cocaine[1]. The production of heroin was outlawed in the United States in 1924 and the last vestiges surrendered to the federal government upon passage of the Narcotic Control Act of 1956.

Herion use continues to be a problem today. In 2003, the National Survey on Drug Use and Health indicated that an estimated 3.7 million individuals had used heroin at some point during their lives. The 2006 report indicated that the number of current heroin users rose by about forty percent between 2005 and 2006 increasing the prevalence rate from 0.06 to .14. The majority of the heroin sold today is smuggled illegally into the United States from countries such as Afghanistan and Burma. In fact, 1/3 of the Afghan economy is based on the production of opium (Department of State, 2008). Studies estimate that heroin use cost the United States between $15 and 20 billion in 1996 (Mark & Juday, 1999). This number includes the cost of criminal

acts committed under the influence of heroin, lost productivity of heroin users and healthcare costs related to the treatment of heroin addiction and other illnesses or accidents related to heroin use. These figures are startling and present a very real and critical rationale for furthering our understanding of heroin and its effects on the heroin user.

The Neurobiology of Opiate Use and Addiction

Heroin has been characterized as a more potent and faster acting opiate than morphine with users reporting an intense 'rush' almost immediatly upon intravenous administration. Following inhalation or injection into the bloodstream, the presence of acetyl groups on the heroin molecule render it lipophilic and facilitate its rapid transportation across the blood brain barrier allowing for these acute physiological effects. Due to its unique chemical structure, 68% of heroin administered intravenously will enter the brain compared with only 5% of morphine administed in the same manner (Oldendorf, 1972; Hartveg et al, 1984). Once within the brain, however, heroin undergoes deacetylation and is converted to its pharmacologically active metabolites morphine and 6-monoacetylmorphine[1]. It is the metabolites of heroin, rather than heroin itself, that create the myriad of symptoms and side effects that are associated with heroin use (Way et al., 1960; Umans & Inturrisi, 1981; Inturrisi et al 1983). For this reason, heroin is considered to be a 'prodrug'. Many of the differences in pharmocodynamics and pharmacokinetics observed between morphine and heroin are attributed to 6-monoacetylmorphine which is formed following heroin deacetylation. Maximal concentrations of plasma 6-

[1] When administered orally, heroin deacetylation will occur in the periphery and the resulting physiological effects will more closely mirror those of morphine (Sawynok, 1986).

monoacetylmorphine are detected in less than three minutes following intravenous heroin administration (Rook et al., 2006) indicating a probable role for this metabolite in the acute physiological effects of heroin. 6-monoacetylmorphine is then further metabolized into morphine within thirty minutes (Umans & Inturrisi, 1981).

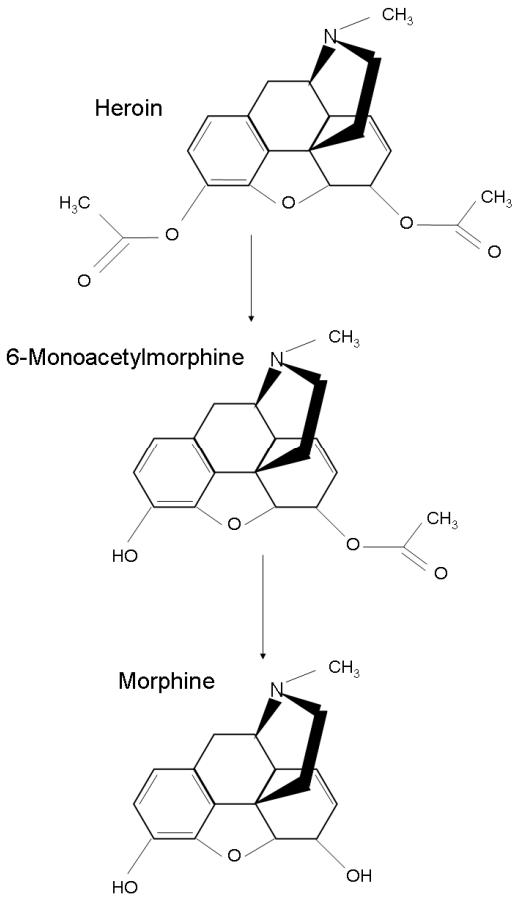

Figure 1.1 Heroin (diacetylmorphine) is rapidly metabolized in the body into 6-monoacetylmorphine which is then further metabolized into morphine.

Opioid drugs exert their physiological effects by binding to opioid receptors either within the central nervous system or in the periphery. Three classes of opioid receptors have been described; mu, delta and kappa. All of the opioid receptors are seven transmembrane G-protein coupled receptors (GPCR). These receptors undergo

a conformational change of the cytoplasmic domain upon the binding of an agonist thus allowing for the heterotrimeric G-protein to dissociate and alter intracellular signalling pathways. The end result of these pathways may include transient changes in neuronal signaling and/or long term alterations in gene expression. The major endogenous ligands of the opioid receptors include the three families of enkaphalin-containing opioid neuropeptides: the endorphins[2], the enkephalins[3] and the dynorphins[4]. The endorphins were originally thought to be the endogenous mu-opioid receptor ligand but in fact these peptides show only limited agonistic activity at the receptor. Further research has revealed another class of opioid neuropeptides, the endomorphins, which preferentailly bind to the mu-opioid receptor with high affinity and specificity (Zadina et al., 1997). Both morphine and 6-monoacetylmorphine are potent exogenous agonists of the mu-opioid receptor for which heroin shows only a slight affinity because of its unique molecular structure. Research suggests that there exists a number of splice variants of the mu-opioid receptor gene which give rise to functional subtypes of the receptor. For example, activity at the mu_1 opioid receptors is associated with the analgesic properties of opioid drugs while activity at mu_2 receptors is responsible for gastrointestinal and respiratory suppression, euphoric feelings and physical dependence (Schwartz, 1998). In addition to the mu-opioid receptor, the agonist activity of 6-monoacetylmorphine at the delta-opioid receptor in both the brain and spinal cord may play a role in heroin-induced analgesia (Rady et al., 1994).

Heroin, as with all drugs of abuse, activates the brain's reward pathway. Research suggests that this activation is the result of disinhibition of GABA

[2] A contraction of 'endogenous morphine'

[3] from the Greek words *en* for 'in' and *kephale* for 'head'

[4] An abbreviation for 'dynamic endorphins'

interneurons in the ventral tegmental area (VTA), possibly via the binding of heroin metabolites to the mu-opioid receptors present on these cells. In support of this, both systemic and intra-VTA morphine administration results in hyperpolarization of interneurons and increased firing of VTA dopamine containing neurons (Johnson & North, 1992). Allison et al. (2006) demonstrated that the GABA interneurons of the VTA communicate via connexin-36 gap junctions allowing for rapid excitatory activity between neighboring cells. Furthermore, it appears that signalling via these gap junctions may be inhibited by activation of their mu-opioid receptors as systemic heroin administration, similar to the application of gap junction blockers, reduces IC-evoked post-stimulus spike discharges of the VTA GABA interneurons (Steffenson et al., 2006). Therefore, the binding of heroin metabolites to mu-opioid receptors on GABA interneurons in the VTA may induce changes in these neurons that result in the inhibition of the gap junctions. This leads to the hyperpolarization of these cells and reduces the inhibitory GABA signal that allows for increased firing of the dopaminergic neurons. As a result of increased VTA dopaminergic neuronal activity, dopamine release in VTA target regions such as the nucleus accumbens and prefrontal cortex is likely to be increased (Kelly et al., 1980; Schultz, 1997). In fact, microdialysis studies demonstrate increased dopamine levels in the nucleus accumbens following the systemic administration of morphine (Rada et al., 1991).

Interestingly, exposure to a stimulus that has become associated with drug use will also result in the firing of the VTA dopaminergic neurons and increased extracellular dopamine within the nucleus accumbens (Fontana et al., 1993). In other words, a formerly neutral stimulus that has been paired with drug administration may induce neural activity similar to that seen upon actual drug administration. This suggests that these drug cues may gain reinforcing properties through a learned

association with administration of the drug. In addition, evidence from behavioral models such as the conditioned place preference test suggest that the VTA-Nacc pathway may play a critical role in drug addiction (Shippenberg et al., 1992). For example, heroin seeking induced by a priming injection of heroin or exposure to stimuli previously associated with heroin use resulted in an increase in glutamate release in the core region of the NAcc. Furthermore, this increase was inhibited by temporary inactivation of synpatic activity in the prelimbic cortex supporting the role of a pathway from the prelimbic region to the Nacc in drug seeking (LaLumiere & Kalivas, 2008). Taken together, these data suggest that the neural circuitry involved in conditioned drug effects may be similar to those mediating the effects of the drug itself.

The Effects of Opioids on Health Status

It is well-documented that clinically relevant opioids, like morphine, exert a profound influence on immune status. Administration of morphine has been associated with alterations in leukocyte proliferation (Roy et al., 1996; Houghtling et al., 2000; Bayer et al., 1990a; Eisenstein et al., 1991), natural killer cell activity (Shavit et al., 1986; Bayer et al., 1990b), T cell differentiation (Roy et al., 2001) inflammatory responses (House et al., 2001) and antibody production (Lockwood et al., 1994). Most importantly, administration of morphine has been shown to disrupt the innate immune system which provides the first line of defense against invading pathogens. If the innate immune response is weakened, pathogens may be allowed to pass more readily into the body and rapidly proliferate. For example, animals treated with morphine exhibit symptoms of increased gastrointestinal permeability, such as

the systemic dissemination of gut flora and orally ingested Salmonella (Hilburger et al., 1997; MacFarlane et al., 2000). Morphine-treated animals also show a decrease in neutrophil chemotaxis and delayed entry of neutrophils into the alveolar compartments after infection with Streptococcus pneumonaie (Grimm et al., 1998; Wang et al., 2008).

In addition, the innate immune system is responsible for the induction of the adaptive immune response and a breakdown in the communication between these two systems may result in an increased vulnerability of the host to damage wrought by the pathogen. For example, both morphine and heroin have been shown to alter the expression of cytokines such as TNF-α and IL-1β both of which are released by cells of the innate immune system and are involved in the facilitation of the adaptive immune response (Clark et al., 2007; Szczytkowski & Lysle, 2007). Studies in our own laboratory have shown that acute morphine administration in rats suppresses NK cell activity, splenic lymphocyte proliferation in response to T- and B-cell mitogens, blood lymphocyte mitogenic responses, and *in vitro* production of the cytokines interleukin-2 and IFN-γ (Fecho et al., 1993; 1996 a,b; Lylse et al., 1993). Our studies further show that the immune alterations induced by morphine are dose-dependent and antagonized by naltrexone, an opioid receptor antagonist (Fecho et al., 1996a). Research has suggested that the effects of opioids on immunity are mediated specifically via the mu-opioid receptor as antagonism of these receptors eliminates the immunomodulation normally seen with morphine administration (Nelson et al, 2000). In addition, several immune alterations normally seen with morphine treatment are not observed in mice lacking the mu-opioid receptor (Roy et al., 1998).

Although there has been extensive research on the effects of morphine on immune status, relatively little is known about the immunomodulatory effects of

heroin. The high prevalence of opportunistic infections reported among heroin users has long suggested that heroin use impairs the ability of the host to combat infectious disease (Luttgens, 1949; Hussey & Katz, 1950; Louria et al., 1967). Moreover, bacterial infections of soft tissue have been shown to account for more than one-third of drug-related hospital admissions, with bacteriologic data indicating that these are typically "mixed" infections (e.g., Orangio et al., 1984; White et al., 1973). Bacterial infections associated with injection drug use can progress to major systemic infection and cause severe damage to tissues such as the heart and lung. Although it has been postulated that this high rate of infection amongst heroin users results from increased contact with pathogens, possibly through needle sharing or impurities in street grade drugs, there is evidence that heroin may directly impact the clinical course of infection (Kreek, 1990; Farizio et al., 1992; McLachlan et al., 1993) and diseases associated with intravenous heroin use may be influenced by the ability of opioids to modulate immune function (McDonough et al., 1980; Donahoe et al., 1986; Novick et al., 1989; Ochshorn et al., 1990). Interestingly, opioids prescribed for analgesia to burn victims and cardiac patients appear to influence susceptiblity to infections as higher doses of opiate drugs have been associated with increased infectious complications in these patients (El Solh et al., 2006; Schwacha et al., 2006). These data suggest that the use of opioids increases the risk of infection despite the mode of administration. In fact, clinical studies report abnormalities in basic immune parameters in heroin users, including decreased numbers of circulating lymphocytes, decreased NK cell activity, and reduced antibody-dependent cellular cytotoxicity (Nair et al., 1986; Govitrapong et al., 1998), suggesting that heroin may further contribute to increased infection susceptibility among users, independently of contemporaneous risk factors. In further

support of this, studies have indicated that human heroin users exhibit poorer immune responses following hepatitis vaccination (Quaglio et al. 2004; Rodrigo et al., 1992).

Opiates appear to elicit immunomodulation via both central and peripheral mechanisms. Research suggests that leukocytes such as dendritic cells (Li et al., 2009), granulocytes and monocytes (Lopker et al., 1980), as well as T-lymphocytes (Mehrishi & Mills, 1983) all exhibit functional opioid receptors through which direct immunomodulation may occur. For example, in vitro phagocytosis performed by macrophages is inhibited by the addition of morphine to the culture and antagonist studies suggest that these effects are mediated via the mu-opioid receptor (Casellas et al., 1991; Szabo et al., 1993). Furthermore, morphine decreases the production of interferon-gamma from Con-A stimulated peripheral blood mononuclear cells and promotes the growth of HIV-1 in culture (Peterson et al., 1987; 1990). Other evidence suggests a central mechanisms through which opiates may impact immune functioning. Shavit et al. (1986) demonstrated that morphine given systemically induced a decrease in natural killer cell activity which was reversed upon naltrexone administration. Interestingly, N-methylmorphine which does not readily cross the blood brain barrier and enter the CNS did not alter the activity of natural killer cells supporting the idea that morphine may be acting centrally to alter immune parameters. In addition, microinjections of opiates directly into the periaqueductal gray of rats induces a profound reduction in natural killer cell activity that is reversed with naltrexone administration (Weber & Pert, 1989). Studies in our own laboratory have shown that intracerebroventricular (icv) administration of morphine dose dependently decreases both lymphocyte proliferation and natural killer cell cytotoxicity in the spleen (Lysle et al., 1996). Furthermore, our studies have shown that N-methylnaltrexone administered icv dose-dependently antagonizes the

immunomodulatory effects of systemic morphine, whereas systemic administration of N-methylnaltrexone (at doses that do not act centrally) is ineffective in blocking morphine's effects (Fecho et al., 1996a). Taken together, these data suggest that opiates may alter immune functioning through both direct and indirect pathways.

The Role of Proinflammatory Mediators in Immunity

Although tremendous progress has been made in the understanding of how opioids influence immune processes, little agreement has been reached about what constitutes an appropriate measure of immune status. The immune system is obviously complex involving a vast number of cell types and an even greater number of factors produced by each cell type, all of which are precisely orchestrated into a cascade of activity. The experiments reported here focus on the conditioned effects of heroin on nitric oxide expression because of the widespread impact that nitric oxide has on immune function. Studies have shown that nitric oxide is produced by many cells of the immune system and that it mediates diverse biological functions including vasodilation, the cytotoxic activity of macrophages, and the inhibition of platelet adhesion and aggregation (Suschek et al., 2004; Tuteja et al., 2004). Nitric oxide is formed from the guanidine-nitrogen of L-arginine by a group of isozymes termed nitric oxide synthases (NOS). Although the nomenclature varies, there are three known NOS genes, neuronal NOS, endothelial NOS, and inducible (iNOS). Among the three NOS isoforms, the neuronal and endothelial genes are constitutively expressed, whereas the expression of the inducible gene is dependent upon external factors such as exposure to pathogens.

Nitric oxide is a gaseous molecule with a very short half-life making it difficult to directly measure. However, nitric oxide production may be measured

indirectly by analyzing the expression of inducible nitric oxide synthase (iNOS), one of the enzymes responsible for the synthesis of nitric oxide. Under normal physiological conditions, iNOS is not expressed by mammalian cells but rather is induced by exposure to proinflammatory stimuli. In the laboratory we utilize lipopolysaccharide (LPS) to induce expression of iNOS and production of nitric oxide. LPS is a component of the gram-negative bacterial cell wall that binds to the Toll-like Receptor 4 (TLR-4) and CD14 on immune cells. Upon LPS binding to these receptors a signaling cascade is induced that initiates the translocation of NF-kappaB to the nucleus of the cell. NF-kappaB then acts to direct the upregulation of certain genes to increase the transcription of iNOS as well as the proinflammatory cytokines, TNF-α and IL-1β.

Immunohistochemical localization of iNOS in rats exposed to LPS shows the presence of the iNOS enzyme in a number of immune cells in a variety of tissues: macrophages, lymphocytes, neutrophils, and eosinophils within the spleen; Kupffer's cells and hepatocytes within the liver; alveolar macrophages within the lung; macrophages in the adrenal gland; and eosinophils and mast cells within the colon (Bandaletova et al., 1993). Most important for the current project, the production of iNOS by macrophages and other cells of the immune system provides a substantial degree of microbial resistance (Breitbach et al., 2006; Green et al., 1990; Hoffmann et al., 2006; Rossi et al., 1999; Vincendeau et al., 1992). Mice lacking the gene for inducible nitric oxide synthase show an increased susceptibility to parasitic and bacterial infections (MacMicking et al., 1995; Murray et al., 1999; Wei et al., 1995). The production of large quantities of nitric oxide by macrophages also provides resistance to viral infections and display tumor cytotoxicity (Green & Nacy, 1993; Hibbs et al., 1987; Karapiah et al., 1993). In addition to its role as an anti-microbial

agent, nitric oxide also serves many immunoregulatory functions. In addition to its role in the control of infectious organisms, nitric oxide produced by iNOS appears to be involved in controlling the onset and duration of cellular immune responses (Fu & Blankenhorn, 1992) For example, there is evidence that nitric oxide suppresses the formation of antibodies to tetanus toxoid and sheep red blood cells following salmonella typhimurium immunization (Al-Ramadi et al., 1992; Eisenstein et al., 1994). Numerous studies have also shown an anti-proliferative effect of nitric oxide on lymphocytes (Albina et al., 1991). Most importantly for the purpose of these studies, work in our laboratory has demonstrated that morphine alters the expression of nitric oxide induced by the Gram negative pathogen, Porphyromonas (Bacteroides) gingivalis.

In addition to effects on nitric oxide, there is evidence that opiate administration may also alter the production of the proinflammatory cytokines, TNF-α and IL-1β (Chao et al 1993; Bencsics et al 1997; Pacifici et al 2000). Similar to iNOS, IL-1β and TNF-α both play critical roles in the body's defense against infectious challenge, while also serving to regulate the immune response to these challenges. Both IL-1β and TNF-α are proinflammatory cytokines produced primarily by activated macrophages. Once secreted, IL-1β elicits widespread effects that range from inducing fever via actions within the hypothalamus to stimulating neutrophil binding along the endothelial wall to allow for infiltration to the site of an infection. IL-1β also stimulates T-cell activation and proliferation; B-cell maturation, proliferation, and immunoglobulin production, as well as the synthesis of other cytokines by immune cells. Interestingly, many of the proinflammatory properties of TNF-α resemble those of IL-1β. In fact, these two cytokines are able to stimulate the production of each other, and are also capable of acting in synergy to stimulate T- and

B-cells. TNF-α was initially discovered based on its ability to induce hemorrhagic necrosis and regression in certain tumors and has since been shown to be involved in a wide range of immune functions. Among its most important functions, TNF-α induces fever, promotes immune cell migration to sites of infection, stimulates T- and B-cells to become active and proliferate, and has been shown to possess nonspecific antiviral properties. In addition, it appears that both TNF-α and IL-1β are highly involved in LPS-induced septic shock. Rats with LPS-induced septic shock exhibited a peak in TNF-α in plasma at one hour post-infection with levels beginning to decline by two hours after LPS administration. Similarly, IL-1β peaked at one hour post-LPS and gradually returned to baseline over nine hours (Lin et al, 2006). Given the critical nature of these proinflammatory mediators in the initial response to infection it is imperative that we understand how heroin and heroin related cues may alter their effectiveness.

Conditioned Immune Alterations

It was originally believed that the immune system functioned in isolation from the rest of the body and was considered to be an autonomous, self-regulating entity responsible for the prevention of infection and the maintenance of health. Recently it has been shown that the cells and processes of the immune system are highly influenced by both internal and external factors that can alter the immune response thereby modulating the susceptibility of the host to pathogens and the progression of disease (e.g., Vishwanath, 1996; Straub & Besedovsky, 2003). Factors such as an individual's psychological state or learned behaviors have been shown to influence the functioning of the immune system. In fact, Pavlovian conditioning of the immune

response is a well-documented phenomenon that can be accomplished with a wide variety of immunomodulatory stimuli providing some of the most concrete evidence for communication between the nervous and immune systems. Research has shown that the pairing of a neutral stimulus that does not evoke changes in immune status (e.g., a sound, taste, smell or specific environment) with a stimulus that is itself immunomodulatory results in the formerly neutral stimulus acquiring immune altering properties. Most investigators would agree that Pavlovian conditioning is an associative learning process by which an organism may become better prepared to adapt its behavior as necessary (e.g., Rescorla, 1988). The Pavlovian conditioning of the immune response may be viewed as a means by which an organism learns the predictive value of a stimulus that has become associated with an immunologically relevant outcome such that it may adapt not only its behavior but the response of its immune system as well. The ability of the organism to learn associations between stimuli predictive of immunological challenge may allow the host to alter immune function in anticipation of these challenges in an attempt to more adequately respond to environmental events.

One of the earliest modern demonstrations of conditioned immunomodulation involved the pairing of the immunomodulatory drug cyclophosphamide with the sweet taste of saccharin. Interestingly, Ader (1974) observed that some of the animals that had received pairings of CY and saccharin died when repeatedly reexposed to the saccharin solution alone. Furthermore, mortality rates for these animals seemed to correlate with the volume of saccharin solution ingested during the conditioning trial. It appeared possible from these results that the taste of saccharin took on immunosuppressive properties of its own through its association with cyclophosphamide and animals exposed to the saccharin solution during testing might

be more susceptible to pathogens in the environment which led to increased mortality rates among these animals. This was confirmed by a study in which rats underwent either 1- or 2- pairings of CY (50 mg/kg) with saccharin solution and three days later were administered an injection of the antigen, sheep red blood cells (SRBC). Interestingly, the experimental groups reexposed to the conditioned stimulus prior to testing not only demonstrated an aversion to the saccharin solution but also exhibited a reduction in anti-SRBC antibody titers compared to the control groups (Ader & Cohen, 1975). The conditioned suppression of anti-SRBC antibodies was still present up to 15 days after challenge with the antigen, evidence that these effects are not transient but long-lasting (Ader et al., 1982; Neveu et al., 1986). The conclusion reached by Ader & Cohen was that the reduction in antibodies was a result of "behaviourally conditioned immunosuppression" brought about by the learned association between the CY and the saccharin solution. These experiments were replicated shortly thereafter by other laboratories who reported similar findings (e.g., Rogers et al., 1976; Wayner et al., 1978). This same principle was used to show that conditioned immunosuppression has the ability to alter the clinical course of autoimmune diseases, such as systemic lupus erythematosus (Ader & Cohen, 1982; Olness & Ader, 1992). Using a different immunomodulatory agent, others have shown a conditioned reduction in the severity of adjuvant-induced arthritis in mice (Lysle et al., 1992). In addition to the conditioning of suppressive effects on immune function much recent research has focused on conditioned immune enhancement. Research has shown that exposure to a stimulus that had previously been paired with antigen results in an increase in antibody titers (Ader et al., 1993; Chen et al., 2004). Taken together, these results provided concrete evidence that conditioned

immunosuppression is possible and suggested that there must be a connection between the nervous and immune systems mediating these effects.

The studies conducted by Ader and Cohen initially, and by others subsequently, have utilized similar experimental designs. The experimental group receives exposure to the immunomodulatory US paired with the neutral CS either repeatedly or in the case of taste-immune associations only one pairing is necessary. At some later point, which varies between experiments, the experimental group is reexposed to the CS without the US and at this time may also receive an immunological challenge. The specificity of the response is assured by the use of a comprehensive set of control groups including a non-conditioned group of animals that are exposed to the CS as well as the US but in a non-contingent manner. These animals are subsequently reexposed to the CS during testing to confirm the lack of an immunological effect of the CS. Other control groups include animals that undergo the conditioning paradigm but are not reexposed to the CS during testing. This group serves as a control for any possible effects of the US that may still be present at the time of testing and for potential immunomodulatory effects of the conditioned procedure itself. A group exposed to the US alone allows for the measurement of the unconditioned effects of the immunomodulatory agent which can then be compared to the conditioned effects observed in the experimental group. Some experiments also include a placebo group which receives exposure to the CS but never the US. Still another set of control groups may be included in which animals receive pre- or post-conditioning exposure to the CS which should attenuate the conditioned response and substantiate the effects as dependent upon associative learning processes. The development and persistence of the CR is dependent on several factors and certain experimental manipulations may produce a weakening or inhibition of this response.

Two of the most widely studied experimental paradigms leading to reduced expression of the CR are extinction and latent inhibition. Extinction is evident when after conditioning has taken place repeated exposure to the CS without the US decreases the CR. Latent inhibition is a process by which repeated non-reinforced exposure to a stimulus prior to conditioning will inhibit the formation of a CR to that stimulus (Lubow & Moore, 1959). Extinction and latent inhibition have been studied extensively within models of conditioning to test hypotheses concerning retroactive and proactive stimulus interference, respectively (Pineno & Miller, 2005). Despite the wealth of literature in this area it is as yet unclear whether all immune responses may be conditioned and research continues to unveil the breadth and complexity of conditioned immunomodulation.

Since the early studies of Ader and Cohen, conditioning of the immune response has been shown in a number of different ways. Early studies in our own laboratory demonstrated that a stimulus previously paired with electric shock treatment will induce a number of immune alterations (Lysle et al., 1988; Luecken & Lysle, 1992; Perez & Lysle, 1995). In addition, conditioning to aversive stimuli such as electric shock was found to be mediated through opioid receptors as evidenced by the ability of naltrexone to block the conditioned response (Lysle et al., 1992; Perez & Lysle, 1997). This led to the study of whether the immunomodulatory properties of exogenous opioids, such as heroin and morphine, could be conditioned to environmental stimuli. Several investigators have shown that many of the physiological and behavioral responses to opioids may be conditioned to previously drug-paired stimuli. For example, environmental stimuli that had previously been paired with morphine administration can elicit such morphine-like effects as hyperthermia when presented in the absence of morphine (Bardo & Valone, 1994;

Eikelboom & Stewart, 1979; Miksic et al., 1975; Schwarz & Cunningham, 1990; Wikler & Pescor, 1967). Similar results have been seen with the conditioning of morphine's analgesic properties such that exposure to morphine-associated stimuli induces a conditioned analgesic response in the absence of drug (Miller et al 1990; Bardo & Valone 1994). There has been increasing evidence from studies conducted on human subjects showing that drug-paired stimuli can cause intense craving, feelings of being 'high', galvanic skin responses, autonomic arousal, and altered neural activity in drug users (Sideroff & Jarvik 1980; Ehrman et al. 1992; Foltin & Haney 2000). Exposure to drug cues is one of the key contributing factors to relapse mainly because these cues induce a wide variety of complex, classically conditioned physical and behavioral responses (Unnithan et al 1992; Derbas et al 2001)..

In line with these studies, our laboratory provided the first evidence that alterations of immune status can be conditioned to environmental stimuli that have been paired with morphine administration (Coussons et al., 1992; 1994a; 1994b). More recently we have demonstrated that heroin's effects on nitric oxide expression may also be conditioned to environmental stimuli. In those investigations, rats received subcutaneous injections of heroin (1 mg/kg) upon placement into a distinct environment. When rats were subsequently re-exposed to the environment without heroin administration the production of nitric oxide was suppressed similar to that seen with heroin administration (Lysle & Ijames, 2002). Overall, these data suggest that the conditioned effects of heroin on nitric oxide involve a learned association between the drug and the environment in which it is delivered and that blocking the acquisition and/or expression of this association may prevent these effects. Current research in the area of drug addiction has focused on the critical task of reducing relapse rates by attempting to curb cue induced drug seeking behavior, however, it is

also imperative to take into account the widespread effects these cues may have on immune functioning and the ability of previous drug users to combat infectious disease while in recovery. These results are important, not only because they provide the first demonstration that immunologic alterations can be conditioned to drug cues paired with heroin administration, but also because they have profound implications for opioid/immune interactions. One key implication is that the host response to opioids will not be static, but will change as the organism learns to predict the administration of the drug on the basis of distinctive stimuli in the environment. These effects need to be factored into the comprehensive treatment of opiate users.

Neural Substrates

Conditioning of the immune response involves the learning of an association between the neutral stimulus (CS, e.g., the conditioning chamber) and the immunomodulatory unconditioned stimulus (US, e.g., the immunomodulatory effects of heroin), such that the CS may take on the immunomodulatory properties of the US. This learned association is similar to many other forms of learning and may involve similar brain structures. Several investigators have demonstrated a role of the basolateral amygdala (BLA) in associative learning. For example, lesions of the BLA block classical eye blink conditioning (Blankenship et al., 2005), inhibit conditioned fear responses to aversive stimuli in rats (Lee et al., 2005), and block acquisition of the motivational value of an appetitive US (Setlow et al., 2002). In addition, learning of an inhibitory avoidance task involves neuronal activity within the BLA (Chang et al., 2005) as does olfactory fear conditioning (Sevelinges et al., 2004) and conditioning of odorant attractiveness in female mice (Moncho-Bogani et al., 2005).

More importantly for the purposes of these studies, the BLA has been implicated in the control of the formation of stimulus-reward associations within models of drug abuse. Conditioning to drug-related cues has become a major focus within the study of drug addiction and relapse. Exposure to previously drug-paired stimuli will reinstate drug seeking behavior in rats who have undergone extinction training (Gracy et al., 2000). However, inactivation of the BLA will block the ability of drug cues to reinstate drug seeking in rats that had been previously taught to self-administer cocaine. Interestingly, this same study did not show an effect of BLA inactivation on self-administration of cocaine suggesting that the BLA is critical for conditioned but not unconditioned reinforcement (Meil & See, 1997). In a similar study, inactivation of the BLA was shown to abolish cue-induced reinstatement of drug seeking in rats taught to administer heroin (Fuchs & See, 2002). Furthermore, animals receiving morphine injections paired with a distinct environment showed greater Fos activation within the BLA when exposed to that environment than animals receiving un-paired injections (Harris & Ashton-Jones, 2003). Similarly, cocaine-associated cues elicited neural activity within the BLA nearly identical to that seen upon intravenous delivery of cocaine in rats taught to self-administer (Carellia et al., 2003). Taken together, these studies provide clear and consistent rationale supporting the investigation of the BLA as part of the neurobiological circuitry involved in the conditioned immune responses to heroin.

Interestingly, dopamine receptors within the BLA have been shown to play a role in learning and memory as well as classical conditioning. Post-training intra-BLA dopamine infusions enhance memory consolidation of an inhibitory avoidance task (LaLumiere et al., 2004). Additionally, Macedo et al. (2007) demonstrated that antagonism of dopamine D_1 receptors within the BLA reduces the expression of

conditioned, but not unconditioned, fear. Dopaminergic signaling within the BLA appears to be particularly important for conditioned responses to drugs of abuse. For example, stimulation of dopamine D_1 receptors in the BLA is necessary for the expression of CS-induced reinstatement of cocaine-seeking behavior in rats (See et al., 2001; Yun & Fields, 2003). Similarly, intra-BLA administration of D-amphetamine potentiates cue-induced cocaine seeking possibly by increasing monoamine tone. In contrast, D_2 receptors in the BLA appear to be involved in the acquisition of CS-US associations that guide subsequent CS-induced cocaine-seeking behavior (Berglind et al., 2006). These findings provide a strong rationale for examining the involvement of dopamine D_1 and D_2 receptors in the BLA on the acquisition and expression of heroin-conditioned immunomodulation.

Goals of the Dissertation

This dissertation aims to further current knowledge regarding opioid-induced immunomodulation, specifically by filling a void in our understanding of the neural circuitry mediating the effects of drug cues on immune processes. Published preliminary data (Lysle & Ijames, 2002) indicate that heroin-induced alterations in nitric oxide production may be conditioned to environmental stimluli that predict drug availability The experiments presented herein extend these finding by elucidating neural mechanisms whereby drug cues may alter the expression and production of certain proinflammatory mediators essential for host defense. The experiments in **Chapter 2** demonstrate that the conditioned alterations in nitric oxide production induced by exposure to previously heroin-paired stimuli are a true form of associative learning as the conditioned response is susceptible to both extinction and latent inhibition. **Chapter 3** reveals that the basolateral amygdala (BLA), an area of the brain which has been implicated in the formation of stimulus-reward associations

within models of drug abuse, is necessary for the expression of the conditioned response. Administration of a combination of the GABA agonists, muscimol and baclofen, directly into the BLA blocked the conditioned suppression of proinflammatory mediators while administration of these same drugs into an adjacent region of the caudate did not alter the response. The experiments outline in **Chapter 4** show that the pharmacological blockade of dopamine, D_1 receptors in the BLA reverses the conditioned suppression induced by exposure to previously heroin-paired stimuli. The administration of the dopamine D_1 antagonist, SCH23390, but not the D_2 antagonist, raclopride, resulted in attenuation of the conditioned effect. These studies are important because they are the first to investigate the neural circuitry involved in the conditioned immune alterations produced by exposure to drug cues. Overall, these findings indicate a need to consider the implications of exposure to drug cues on the immune functioning of current and recovering heroin users.

CHAPTER 2

CONDITIONED EFFECTS OF HEROIN ON NITRIC OXIDE EXPRESSION ARE SUSCEPTIBLE TO EXTINCTION AND LATENT INHIBITION

Introduction

The high prevalence of opportunistic infections among heroin users has long suggested that heroin use may alter resistance to infectious disease (Luttgens 1949; Hussey and Katz 1950; Louria et al. 1967; Garten et al. 2004). Although there has been speculation that this high rate of infection may be due to non-sterile intravenous administration of drug, there is increasing evidence suggesting that opiates directly alter immune status (Brown et al. 1974; McDonough et al. 1980; Nair et al. 1986; Donahoe et al. 1986; Novick et al. 1989; Oschorn et al. 1990; Govitrapong et al. 1998; Sharp et al. 2001). Studies in our laboratory have shown that heroin induces alterations in a number of immunological parameters including alterations in natural killer cell activity, T- and B-lymphocyte proliferation, and nitric oxide production (Fecho et al. 2000; Lysle and How *2000*). The reduction in nitric oxide production induced by heroin administration is dose-dependent and appears to be mediated through opioid receptors, as these alterations are reversed upon administration of the opioid antagonist, naltrexone (Lysle and How 2000). The production of nitric oxide by immune cells, particularly macrophages, provides a substantial degree of microbial resistance (Green et al. 1990; Nathan and Hibbs 1991; Vincendeau et al. 1992; Rossi et al. 1999). Mice lacking the gene for inducible nitric oxide synthase show an

increased susceptibility to parasitic and bacterial infections (MacMicking et al. 1995; Wei et al. 1995; Lindgren et al. 2004). The production of large quantities of nitric oxide by macrophages also provides resistance to viral infections and displays tumor cytotoxicity (Karupiah et al. 1993; Chang et al. 2003; Hrabak et al. 2006). In addition to its role as an anti-microbial agent, nitric oxide also serves many immunoregulatory functions. There is evidence that nitric oxide suppresses the formation of antibodies to tetanus toxoid and sheep red blood cells after *Salmonella typhimurium* immunization (Al-Ramadi et al. 1992; Eisenstein et al. 1994). Numerous studies have also shown an anti-proliferative effect of nitric oxide on lymphocytes (Albina and Henry 1991). Thus, nitric oxide plays a critical role in immune processes and may be involved in the altered susceptibility to infection evident in heroin users.

Although it is now apparent that heroin impairs immune status, there has been limited research on how conditioned drug cues may also induce changes in immune function. Classical or Pavlovian conditioning is a well-characterized learning phenomenon in which a formerly neutral stimulus (the conditioned stimulus [CS], such as the conditioning chamber) will begin to elicit a response (the conditioned response [CR]) after repeated pairing with a biologically relevant stimulus (the unconditioned stimulus [US], such as heroin) that had elicited a response upon its first presentation (Pavlov, 1927). Several investigators have shown that many of the physiological and behavioral responses to drugs of abuse may be conditioned to previously drug-paired stimuli. In line with these studies, our laboratory has recently provided new data indicating that heroin's effects on nitric oxide expression may be conditioned to environmental stimuli. In those investigations, rats received subcutaneous injections of heroin (1 mg/kg) upon placement into a distinctive environment which served as the CS. When rats were subsequently re-exposed to the

environment without further heroin administration the production of nitric oxide was suppressed similar to that seen with heroin administration (Lysle & Ijames, 2002). These data provided the first evidence that heroin-induced alterations in nitric oxide expression may be conditioned to environmental stimuli.

The development and persistence of the CR is dependent on several factors and certain experimental manipulations may produce a weakening or inhibition of this response. Two of the most widely studied experimental paradigms leading to reduced expression of the CR are extinction and latent inhibition. Extinction is evident when after conditioning has taken place repeated exposure to the CS without the US decreases the CR. Latent inhibition is a process by which repeated non-reinforced exposure to a stimulus prior to conditioning will inhibit the formation of a CR to that stimulus (Lubow & Moore, 1959). Extinction and latent inhibition have been studied extensively within models of conditioning to test hypotheses concerning retroactive and proactive stimulus interference, respectively (Pineno & Miller, 2005). The susceptibility of conditioned heroin-induced immune alterations to the effects of extinction and latent inhibition demonstrate that this conditioning paradigm is a true form of associative learning and adheres to accepted principles of learning. Furthermore, given the important health consequences that may result from conditioned immune alterations the assessment will identify procedures that influence the CR.

Given this information, it was hypothesized that both pre- and post-exposure to the drug-paired environment (i.e., the CS) should reduce the conditioned effects of heroin on nitric oxide production. To test this hypothesis, the present study evaluated the effects of two behavioral manipulations, pre- and post-exposure to the CS, on the conditioned suppression of iNOS. Rats received five training sessions in which

heroin was administered immediately upon placement into a standard conditioning chamber which served as the CS. In the first experiment, the experimental group [Extinction(Ext)] was exposed to the conditioning chamber without drug for 60-min on each of ten consecutive days following the final conditioning session to induce extinction. In the second experiment, the experimental group [Latent Inhibition (LI)] received ten consecutive days of 60-min non-reinforced pre-exposure to the chamber to induce latent inhibition. Three control groups were included to ensure that the observed results were due to the behavioral manipulations employed. An additional unmanipulated comparison group was included in which the animals received no exposure to the conditioning chambers and no drug treatment. On the test day, the animals were re-exposed to the chamber for 60-min without drug administration and given a subcutaneous injection of LPS (1000 μg/kg) upon removal. Previous research in our laboratory has shown that heroin induces a dose dependent reduction in LPS-induced iNOS mRNA expression in the spleen, lung, and liver. The findings reported here are important because they indicate that previously heroin-associated environmental stimuli are not only capable of inducing alterations in nitric oxide expression but that these effects may be modified by manipulations of the relationship between the environment and drug delivery. These results further implicate a role for associative learning processes in the conditioned effects of heroin on nitric oxide and suggest that these effects may be mediated, at least in part, by centrally located neural substrates of learning.

Materials and Methods

Animals

Male Lewis rats, weighing 225-250 g, were purchased from Charles River Laboratories (Raleigh, N.C., USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12h) cycle maintained through artificial illumination. The animals were allowed access to food and water ad libitum throughout the experiment. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the University of North Carolina at Chapel Hill and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD) and dissolved in 0.9% sterile saline. Animals received a subcutaneous injection of heroin at 1 mg/kg immediately before placement in the conditioning chamber on each of the five conditioning trial days. This dose was selected based on prior experiments in our laboratory showing that a 1 mg/kg dose of heroin alters LPS-induced iNOS mRNA expression and induces conditioning (Lysle and How 2000; Lysle and Ijames 2002). A subsequent study used 3.0 or 0.3 mg/kg heroin doses. The 0.3 and 3.0 mg/kg doses were included to investigate the effect of altering the dose of heroin administered during conditioning on the expression of extinction and latent inhibition. This study also included a control group that was injected with saline-vehicle during conditioning.

Conditioning Procedures

To condition heroin's effects on iNOS expression, all animals received five 60-min training sessions in which they received a subcutaneous injection of heroin upon placement into a standard conditioning chamber. Training sessions were separated by 48 h. The conditioning chambers (BRS/LVE, Laurel, MD, USA) were contained in a room separate from the animal colony and contained a metal grid floor design and cedar bedding to create an environment distinct from that of the home cage. All conditioning took place during the dark phase of the light cycle, and the conditioning chambers were kept dark. The test day took place 10 days after the final conditioning session. Animals were placed back into the conditioning chambers without administration of heroin. After 60 min, the animals were removed from the chambers and given a subcutaneous injection of LPS (1,000 μg/kg) to induce iNOS production. Six hours after LPS administration, all animals were killed, and samples of spleen, liver, and blood were collected for analysis. The 6-h time point was selected based on previous research in our laboratory showing maximal iNOS induction at 6 h after LPS administration (Lysle and How 2000).

Extinction

This study tests whether post-conditioning exposure to a heroin-paired environment without further drug administration would attenuate the conditioned effects of heroin on nitric oxide production. In this experiment, rats were assigned to one of five groups. A schematic representation of these treatment groups is shown in Table 2.1. Four of the groups underwent conditioning, during which, rats received an injection of heroin (1 mg/kg) upon placement into the conditioning chamber. Two of the conditioned groups [extinction (Ext) and extinction/control (Ext/Ctl)] were then

subjected to the extinction procedure, whereas the other two groups remained in their home cages [conditioned (Cond) and conditioned/control (Cond/Ctl)]. The extinction procedure consisted of ten consecutive days of exposure to the conditioning chambers for 1 h a day without administration of heroin. The test day took place on the day after the completion of the extinction procedure just described. On the test day, two groups [one group that had received extinction (Ext) and one group that had not (Cond)] were re-exposed to the conditioning chambers (i.e., the conditioned stimulus [CS]), whereas the other groups remained in their home cages. Re-exposed animals were placed in the chambers for 1 h without heroin and received an injection of LPS (1,000 μg/kg) upon removal. All remaining animals also received an LPS injection at this time. Extinction control (Ext/Ctl) and conditioning control (Cond/Ctl) groups were used to control for any ancillary effects of the extinction and conditioning procedures, respectively. These groups underwent the conditioning protocol but were not re-exposed to the chamber on test day. The fifth group (HC) received no conditioning and remained in the home cage throughout the duration of the experiment, serving as a general control.

Table 2.1 Treatment groups: Extinction and Latent Inhibition Experiments

Extinction:	Days 1-9	Days 10-20	Test Day
HC			LPS
Cond	Conditioning		CS/LPS
Ext	Conditioning	Extinction	CS/LPS
Ext/Ctl	Conditioning	Extinction	LPS
Cond/Ctl	Conditioning		LPS
Latent Inhibition:	**Days 1-10**	**Days 11-19**	**Test Day**
HC			LPS
Cond		Conditioning	CS/LPS
LI	Pre-Exposure	Conditioning	CS/LPS
LI/Ctl	Pre-Exposure	Conditioning	LPS
Cond/Ctl		Conditionoing	LPS

Latent Inhibition

This study tests whether pre-exposure to the conditioning chamber would create a latent inhibition of the conditioned effects of heroin on iNOS expression. As with the previous experiment, rats in this experiment were assigned to one of five groups. A schematic representation of the treatment groups is shown in Table 2.1. Four of the groups underwent conditioning, during which, rats received an injection of heroin (1 mg/kg) upon placement into the conditioning chamber. Two of the groups that would receive conditioning [latent inhibition (LI) and latent inhibition/control (LI/Ctl)] underwent the latent inhibition procedure before the start of conditioning, whereas the other two groups remained in the home cages. The latent inhibition procedure consisted of ten consecutive days of exposure to the conditioning chambers before the first conditioning session. These pre-exposures lasted for 1 hour, and no drug was given at this time. On the day after the final pre-exposure, the rats were subjected to the conditioning procedures described above. On the test day, two groups [one that had undergone pre-exposure (LI) and one group that had not (Cond)] were re-exposed to the conditioning chambers, whereas the other animals remained in the home cage. Re-exposed animals received an injection of LPS (1,000 μg/kg) upon removal from the chambers. All remaining animals also received an LPS injection at this time. Latent inhibition control (LI/Ctl) and conditioning control (Cond/Ctl) groups were used to control for any ancillary effects of the pre-exposure and conditioning procedures, respectively. These groups underwent the conditioning protocol, but were not re-exposed to the chamber on test day. A fifth group (HC) received no conditioning and remained in the home cage throughout the duration of the experiment, serving as a general control.

Dose Dependency

In this set of experiments, we sought to determine the effects that altering the dose of heroin given during conditioning might have on the expression of extinction or latent inhibition. During the conditioning phase of the experiment, animals received five sessions in which an injection of 0, 0.3, or 3.0 mg/kg heroin was given upon placement into the conditioning chambers. For each experiment, rats were assigned to one of six groups. For the extinction experiment, one group of rats from each of the three conditioning doses of heroin was subjected to the extinction procedure. After the last conditioning session, the extinction groups were given ten consecutive days of 1 h exposures to the conditioning chambers. For the latent inhibition experiment, one group of rats from each of the three conditioning doses of heroin was subjected to the latent inhibition procedure. For ten consecutive days before the first conditioning session, the latent inhibition groups were pre-exposed to the conditioning chambers for 1 h each day. There was no drug given during the extinction or the latent inhibition sessions. On test day, all animals were re-exposed to the conditioning chambers. Upon removal from the chambers, all animals received an injection of LPS (1,000 μg/kg).

Real Time RT-PCR

To determine iNOS expression, real time RT-PCR was performed on tissue samples from the spleen and liver. Total RNA was extracted from a section of each of the tissues using TRI-Reagent (Molecular Research Center, Cincinnati, OH), a modification of the original method described by Chomczynski and Sacchi (1987). RNA was quantified spectrophotometrically (GeneQuant II, Pharmacia-Biotech, Piscataway, NJ, USA). For the RT-PCR, reverse transcription is performed using

Oligo(dT)$_{18}$ primer and Moloney Murine Leukemia Virus-Reverse transcriptase following the protocol of the Advantage RT-for-PCR Kit from Clontech (Palo Alto, CA, USA).

PCR amplifications were performed using the Fast Start^{TM} DNA Master SYBR Green I Real-Time PCR Kit (Roche) and the LightCycler instrument (Roche). A master mix containing all reaction components was prepared for all reactions, with each reaction using a 20 ml mix placed in glass capillary tubes specifically designed for use in the LightCycler system. The PCR primer set for iNOS, 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGGTGTCAGA-GTCTTGTGCCTTTGG-3' was synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, UNC-Chapel Hill). Copy numbers were generated from an external standard curve. Amplifications were carried out for 40 cycles and curves showing fluorescence at each cycle were determined by the computer software (Roche). Samples were pre-incubated for 10 minutes at 95^{o} C to activate the Fast-Start Taq DNA polymerase. The cycle temperatures were 95, 60, and 72^{o}C for the denaturing, annealing, and extending, respectively. The cycle times were 15, 5, and 25 s for the denaturing, annealing, and extending, respectively. Flourescence level was determined at the end of the extending phase for each cycle of PCR. The analysis of the fluorescence level in standards and samples over the course of 40 cycles was used to derive the number of copies of the target molecule in each sample. Additionally, assessments of housekeeping gene expression, cyclophilin, were made to assure comparable quality of RNA among samples. The sequence of the cyclophilin primers was 5'-CCAAGACTGAGTGGCT-3' and 5'-AGATTACAGGGTATTGCG-3'. The data are expressed as a copy number of iNOS (per 10ng cDNA) based on the standard curve using the Lightcycler software (Roche).

Furthermore, to confirm the nature of amplification product, a melt curve analysis was conducted after the final PCR cycle. This analysis involved denaturing the products by slowly heating them to 95^{o}C, during which fluorescence is continuously measured.

Nitrite/Nitrate Assay

The level of nitrite/nitrate in plasma samples was assessed using the Greiss reagent assay. Nitrate and nitrite are formed non-enzymatically when nitric oxide is exposed to oxygen, thus plasma levels of these products indicate the level of nitric oxide production. Total nitrite/nitrate levels is determined by the conversion of nitrate to nitrite utilizing nitrate reductase in the presence of NADPH and flavin adenine dinucleotide, and then an assessment using Greiss reagent. Briefly, 6 μl of plasma diluted in 44 μl of dH2O is incubated for in the dark for 90-min with 10 μl of nitrate reductase (1.0 unit/ml), 20 μl of a 0.31 M phosphate buffer (ph 7.5), 10 μl of 0.86 mM NADPH (Sigma), and 10 μl of a 0.11 mM flavin adenine dinucleotide in individual wells of a 96-well plate. Then, 200 ml of Griess reagent consisting of a 1:1 (v/v) solution 1% sulfanilamide in 5.0% phosphoric acid and 0.1% N-(1-napthyl)ethyl-enedamine dihydrochloride in distilled water was added to the samples. The color developed for ten minutes at room temperature after which the absorbance was determined using a spectrophotometer set at 550 nm. All reactions were carried out in triplicate. The total micromolar concentration of nitrite is determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% using this assay.

Statistical Analysis

The data from the initial extinction and latent inhibition studies were analyzed by one-way analysis of variance (ANOVA) followed by planned comparisons to compare all groups to the unmanipulated control. The data from the heroin dose studies were analyzed by two-way ANOVA, with the first factor set as pre- or post- exposure and the second factor as dose. For the extinction experiment, planned comparisons of the extinction group to the conditioned group were conducted at each of the three heroin doses. For the latent inhibition experiment, planned comparisons of the latent inhibition group to the conditioned group were conducted at each of the three heroin doses. All analyses were conducted with the level of significance set at $p<0.05$.

Results

Extinction

The first set of experiments examined whether repeated post-conditioning exposure to the previously drug-paired environment (i.e., the conditioned stimulus [CS]) would alter the conditioned effects of heroin on nitric oxide production. Figure 2.1 shows the effects of the extinction procedure on LPS-induced expression of iNOS mRNA in spleen and liver tissue.

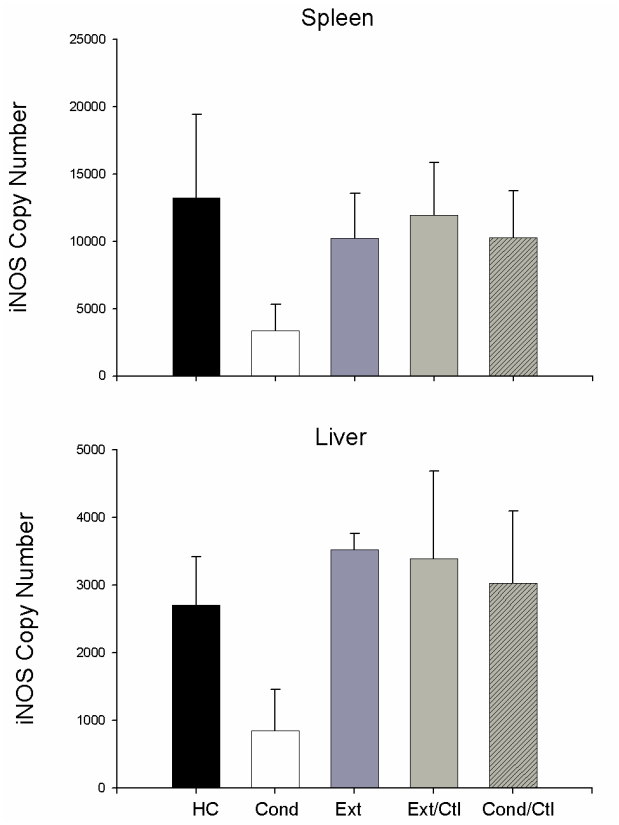

Figure 2.1: The extinction procedure attenuates heroin-induced conditioned suppression of LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. The data are expressed as iNOS copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. The error bars represent the standard error of the mean.

Analysis of iNOS copy number in spleen and liver revealed a significant main effect of treatment [$F(4,15)=3.60$, $P<0.05$; $F(4,15)=6.96$, $P<0.01$]. Moreover, the group that underwent conditioning and was re-exposed to the conditioning chamber on test day (Cond) exhibited a significant reduction in iNOS mRNA as compared to the home cage control (HC) group. This significant difference was evident in both spleen and liver tissue ($P<0.05$) which supports our earlier findings indicating that exposure to a previously heroin-paired environment reduces the expression of iNOS mRNA. Most importantly, there were no significant differences between the HC group and the extinction (Ext) group demonstrating that repeated exposure to the drug-paired environment after conditioning attenuates the conditioned response. Furthermore,

there were no significant differences between the HC group and any of the control groups indicating that these results are specific to the behavioral manipulations and are not due to ancillary effects of the conditioning or extinction procedures. As shown in Table 2.2, there were also no differences found in expression of the housekeeping gene, cyclophilin, between any of the groups indicating that the alterations in mRNA were specific for iNOS and not the result of an overall reduction in RNA expression or production.

Table 2.2
Cyclophilin copy numbers in the spleen and liver as determined by real time RT-PCR Extinction

Group:	**Spleen**	**Liver**
HC	515849(±172651)	110982(±30772)
Cond	539189(±140745)	103034 (±19144)
Ext	586327 (±74685)	152554 (±25171)
Ext/Ctl	553067 (±106493)	101224 (±27674)
Cond/Ctl	724616 (±126050)	109251 (±37130

The data are expressed as mean cyclophilin copy number (±SEM) per 10 ng cDNA sample based on a standard curve using Roche LightCycler software. No differences between treatment groups.

The data in Fig. 2.2 show the effects of each procedure on serum nitrite/nitrate levels. The ANOVA revealed a significant main effect of procedure [$F(4,15) = 7.83$, $P<0.01$] on the levels of nitrite/nitrate in the serum. Planned comparisons showed a significant difference between the HC group and the Cond group [$F(1,15) = 24.21$, $P<0.001$), further supporting our earlier findings that exposure to a previously heroin-paired environment lowers the levels of nitrite/nitrate in the serum, indicating a reduction in nitric oxide production. There were no significant differences between the HC group and the Ext group indicating that the extinction procedure attenuated the conditioned response. There were also no differences between the HC group and

any of the control groups indicating that the results are specific to the extinction and conditioning procedures. The levels of serum nitrate reported here may result from a variety of sources the individual contributions of which are not distinguished. Whereas iNOS is one source of serum nitrate, there are several other factors that may contribute, including endothelial nitric oxide synthase and dietary sources. The data presented below represent an overall alteration in nitric oxide production.

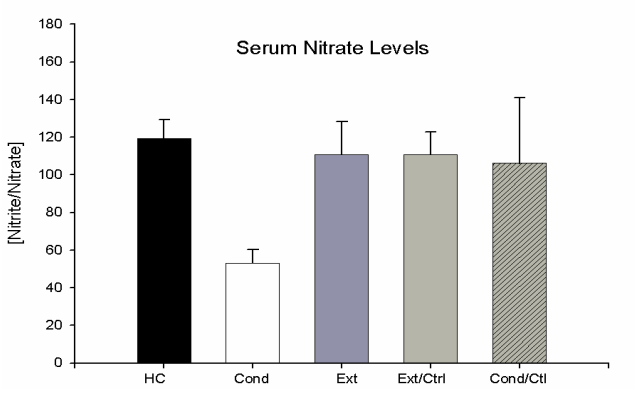

Figure 2.2: The extinction procedure attenuates heroin-induced conditioned suppression of LPS-induced expression of serum nitrate levels as determined by the Greiss Reagant Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent the standard error of the mean.

Latent Inhibition

To test whether a latent inhibition of the conditioned effects of heroin of iNOS could be induced, rats were given exposure to the conditioning chambers before the start of conditioning. Figure 2.3 shows the effects of the latent inhibition procedure on LPS-induced iNOS expression in the spleen and liver. Analysis revealed an overall effect of

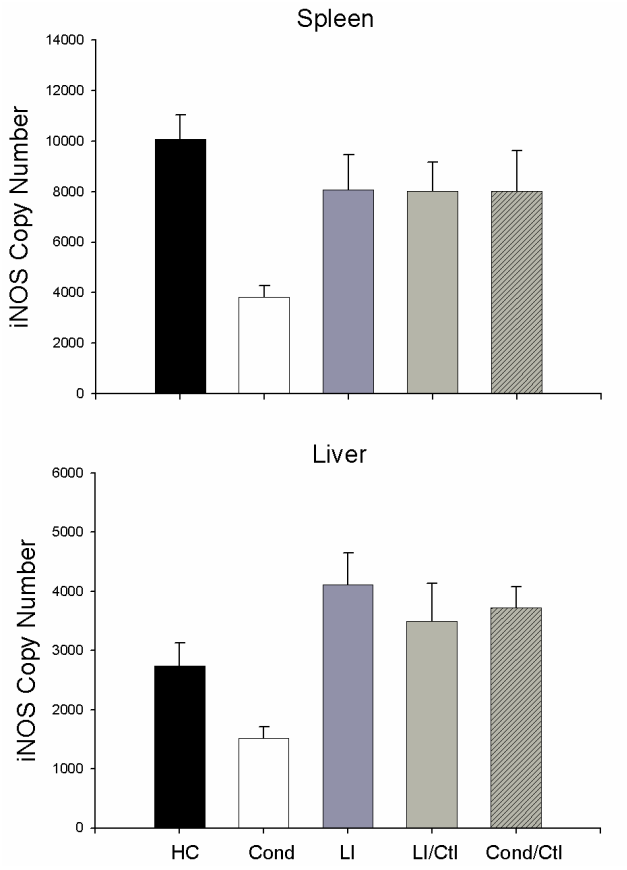

Figure 2.3 Effect of latent inhibition procedure on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. The data are expressed as the mean iNOS copy number per 10 ng cDNA sample based on a standard curve using Roche LightCycler software. The error bars represent the standard error of the mean.

treatment on iNOS copy numbers in the spleen and liver [F(4,15)=3.73, $P<0.05$; F(4,15)=3.65, $P<0.05$]. Planned comparisons showed a significant reduction in iNOS levels in both spleen and liver tissue in the Cond group as compared to the HC group [$F(1,15) = 13.91$, $P<0.005$; $F(1,15) = 16.0$, $P<0.01$). Again, these findings are in line with our earlier reports indicating that exposure to a previously heroin-paired environment will reduce the expression of iNOS mRNA. There are no significant differences between the HC group and the latent inhibition (LI) group indicating that pre-exposure to the drug-paired environment was able to reduce the conditioned effects of heroin on iNOS production. Furthermore, there were no differences

between the HC group and any of the control groups. To ensure that these alterations were specific to iNOS mRNA production and not the result of an overall reduction in RNA processing, real-time RT-PCR was performed on the housekeeping gene, cyclophilin. There were no significant differences between any of the experimental groups in cyclophilin mRNA copy numbers. Table 2.3 shows that there was no effect of group on cyclophilin mRNA copy numbers.

Table 2.3
Cyclophilin copy numbers in the spleen and liver as determined by real time RT-PCR

Latent Inhibition

Group:	**Spleen**	**Liver**
HC	756161(±138641)	128400(±23453)
Cond	755821(±110264)	178192 (±46869)
Ext	826480 (±70957)	175650 (±37319)
Ext/Ctl	658687 (±191141)	163542 (±27482)
Cond/Ctl	781717 (±144229)	161625 (±16680)

Figure 2.4 shows the effects of the latent inhibition procedure on the concentration of nitrite/nitrate in the serum. Analysis revealed a main effect of procedure on nitrite/nitrate levels [$F(4,19)=3.63$, $P<0.05$]. In line with our previous experiments, the Cond group exhibited significantly lower levels of serum nitrite/nitrate than the HC group [$F(1,15) = 41.09$, $P<0.001$)]. There were no significant differences between the HC group and the LI group, again demonstrating that pre-exposure was able to attenuate the conditioned response. There were also no differences found between the HC group and any of the control groups.

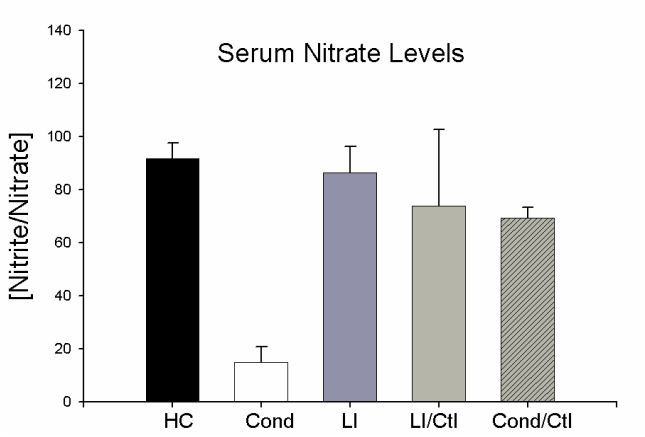

Figure 2.4 Effect of latent inhibition procedure on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent the standard error of the mean.

Effect of Dose

In the following set of experiments, we sought to determine the effects that altering the dose of heroin given during conditioning might have on the expression of extinction or latent inhibition. Figure 2.5 shows the effects of heroin conditioning dose on the expression of extinction. The analysis of iNOS copy number revealed a significant main effect of extinction in both the spleen and the liver, respectively [$F(1,18) = 14.7$, $P<0.01$; $F(1,18) = 5.08$, $P<0.05$)]. There was also a significant dose by extinction interaction within the spleen [$F(2,18) = 8.72$, $P<0.005$]. Planned comparisons revealed that both the 3 mg/kg conditioned and the 0.3 mg/kg conditioned groups showed significantly lower iNOS copy numbers in the spleen [$F(1,18) = 15.29$, $P<0.005$; $F(1,18) = 15.37$, $P<0.005$)] and liver [$F(1,18) = 5.15$, $P<0.05$; $F(1,18) = 5.06$, $P<0.05$)] when compared to the extinction group at the same dose. These data indicate that the extinction procedure was able to block the conditioned suppression of iNOS in both the spleen and liver when either 0.3 or 3.0 mg/kg of heroin was used during the conditioning phase of the experiment.

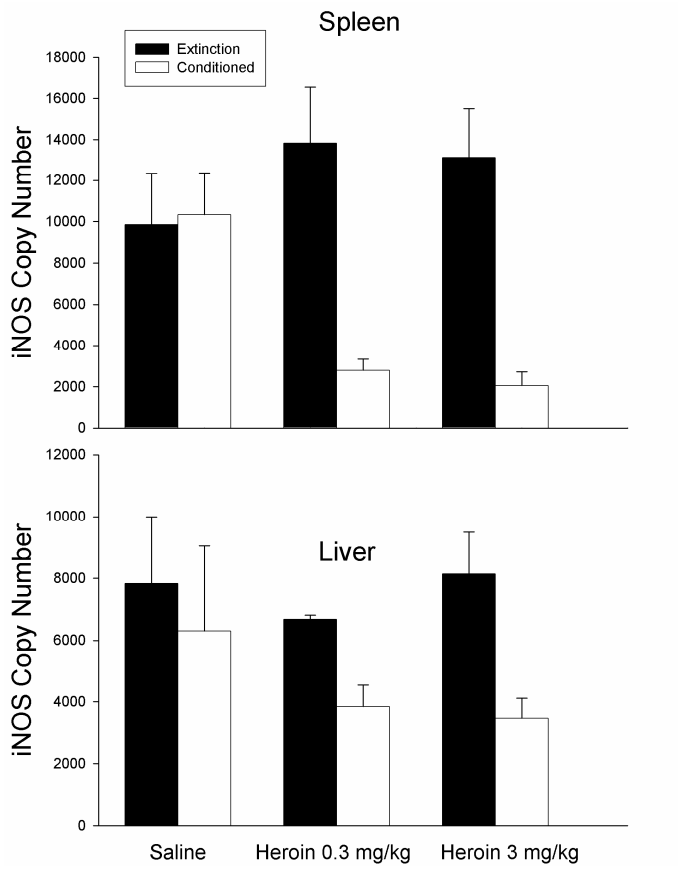

Figure 2.5 Effect of extinction and dose on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. The data are expressed as the mean iNOS copy number per 10 ng cDNA sample based on a standard curve using Roche LightCycler software. The error bars represent the standard error of the mean.

Figure 2.6 shows the effect of heroin conditioning dose on the extinction of LPS-induced nitrite/nitrate production. The assay revealed a significant main effect of extinction [$F(1,18) = 22.38$, $P<0.001$] on nitrite/nitrate levels in the blood and a dose by extinction interaction [$F(2,18) = 7.72$, $P<0.01$]. Moreover, there was a significant reduction in nitrite/nitrate levels in both the 3 and 0.3 mg/kg conditioned groups as compared to the extinction groups conditioned at the same heroin dose [$F(1,18) = 22.09$, $P<0.0005$; $F(1,18) = 14.59$, $P<0.005$]. Thus, the conditioned alteration in iNOS was attenuated by the extinction procedure at both the high and low heroin doses.

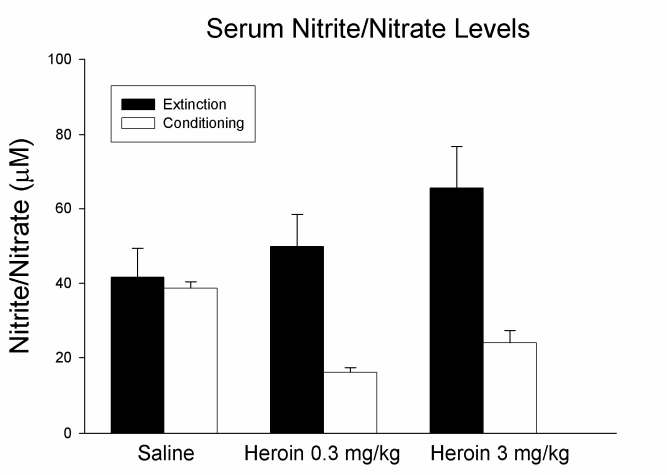

Figure 2.6 Effect of extinction and dose on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent the standard error of the mean.

Figure 2.7 shows the effects of heroin dose on the susceptibility of conditioned immunomodulation to latent inhibition. The analysis of iNOS copy number revealed a significant main effect of dose in the spleen and liver, respectively [$F(2,18) = 11.38$, $P<0.01$; $F(2,18) = 11.12$, $P<0.01$]. Moreover, the 0.3 mg/kg conditioned group showed significantly lower iNOS copy numbers in the spleen [$F(1,18) = 7.34$, $P<0.05$] and liver [$F(1,18) = 7.18$, $P<0.05$] when compared to the 0.3 mg/kg latent inhibition group. The groups treated with 3 mg/kg showed suppressed iNOS levels compared to the saline control groups in both the spleen and liver, respectively [$F(1,18) = 20.07$, $P<0.001$; $F(1,18) = 19.65$, $P<0.001$]. These data show that the latent inhibition procedure attenuated the conditioned suppression of iNOS only at the 0.3-mg/kg dose of heroin.

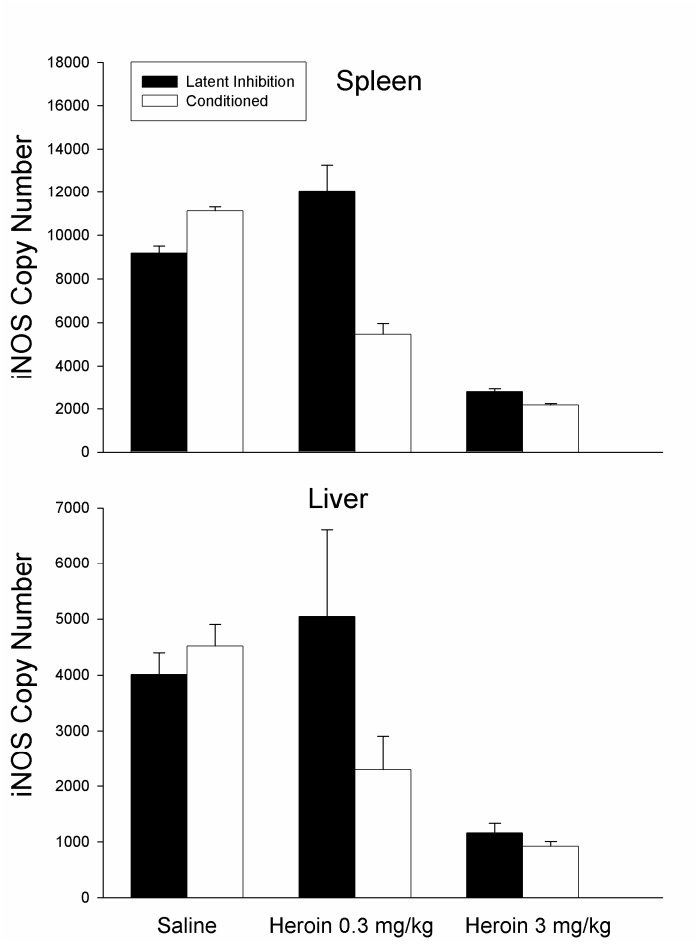

Figure 2.7 Effect of latent inhibition procedure and dose on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. The data are expressed as the mean iNOS copy number per 10 ng cDNA sample based on a standard curve using Roche LightCycler software. The error bars represent the standard error of the mean.

The data in Figure 2.8 show the effect of heroin conditioning dose on the induction of latent inhibition of LPS-induced nitrite/nitrate production. The assay revealed a significant main effect of dose, a main effect of latent inhibition, and a dose by latent inhibition interaction, respectively [$F(2,18) = 7.23$, $P<0.001$; $F(1,18) = 8.1$, $P<0.05$; $F(2,18) = 7.86$, $P<0.005$]. Moreover, there was a significant reduction in nitrite/nitrate levels in the 0.3 mg/kg conditioned group as compared to the 0.3 mg/kg extinction group [$F(1,18) = 22.47$, $P<0.001$]. In addition, the 3.0 mg/kg groups showed reduced nitrite/nitrate levels as compared to the saline control groups [$F(1,18) = 14.44$, $P<0.005$). These data reveal that the conditioned suppression of nitric oxide

production was attenuated by the extinction procedure only at the 0.3-mg/kg heroin dose.

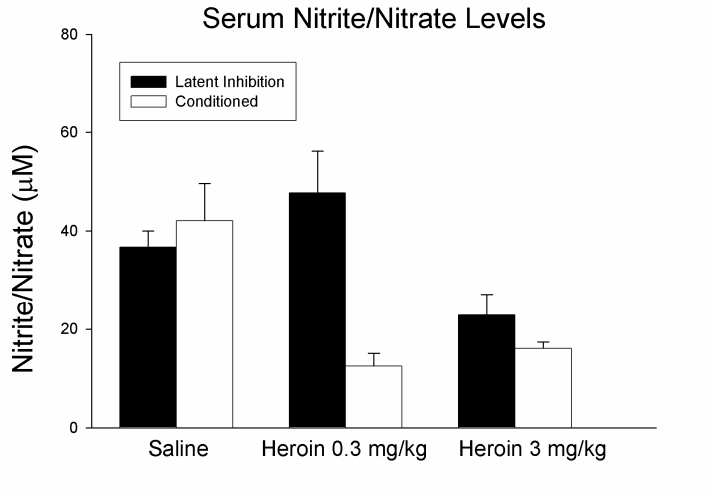

Figure 2.8 Effect of latent inhibition procedure and dose on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The *error bars* represent the standard error of the mean.

Discussion

The results presented here provide the first evidence that exposure to a drug-paired environment, either before or after conditioning, can reduce or even eliminate the conditioned effects of heroin on nitric oxide induction. This effect appears to be widespread as it is found in both spleen and liver tissue as well as serum levels of nitrite/nitrate. The use of several control groups further demonstrates that these results are specific to the manipulations and not related to ancillary effects of the conditioning procedures. These results are important because they provide the first evidence that the conditioned effects of heroin on nitric oxide induction follow accepted principles of learning as they are susceptible to both extinction and latent inhibition. These findings are consistent with prior investigations showing that

heroin-induced alterations in immune status may be conditioned to drug-paired stimuli (Lysle & Ijames, 2002). In this investigation, animals received subcutaneous injections of heroin upon placement in a distinctive environment. Upon re-exposure to that environment in the absence of drug the subjects exhibited a reduction in nitric oxide production similar to what is observed with actual heroin treatment. In a similar study, animals administered cocaine were found to exhibit decreased splenocyte proliferation, reduced spleen weights and an inhibition of IL-10 production and these same immune alterations were evident following drug-free exposure to previously cocaine-paired stimuli (Kubera et al., 2008).

The present study is consistent with these results and reconfirmed the ability of environmental cues to induce heroin-like alterations in immune parameters. Furthermore, these we were able to demonstrate that manipulations involving the animal's experience with the conditioned stimulus will alter the conditioned effects of heroin on iNOS production. In these experiments, animals repeatedly received injections of heroin (1 mg/kg) immediately upon placement into the conditioning chamber. As expected, placement into the chamber without further drug administration was able to elicit reduction in LPS-induced nitric oxide production similar to that seen upon actual heroin administration. Furthermore, both pre- and post-conditioning exposure to the conditioned stimulus (i.e., conditioning chamber) significantly reduced the conditioned response. In order to determine the effects of heroin dose on the susceptiblity of the conditioned response to extinction and/or latent inhibition a second set of studies was conducted in which the dose of heroin given during conditioning trials was varied. In the extinction dose experiments, the extinction procedure was able to attenuate the conditioned reduction in iNOS in both the 3.0 and 0.3 mg/kg heroin groups. However, the latent inhibition procedure was

only able to lessen the reduction in the 0.3 mg/kg group. This suggests that the high dose of heroin may overpower the pre-exposure parameters used in this experiment by providing a more salient stimulus. It is possible that extending the number of days on which animals receive pre-exposure may overcome the potency of the higher dose heroin stimulus.

It is well documented in the literature that a drug-paired environment or stimulus may elicit a response similar to that observed upon drug administration. Repeated pairing of drugs that activate reward pathways with environmental stimuli may lead to these stimuli acquiring secondary reinforcing properties and eliciting drug-like responses. For example, drug-paired stimuli has been shown to cause intense craving, feelings of being 'high', galvanic skin responses, autonomic arousal, and altered neural activity in drug users (Sideroff & Jarvik 1980; Ehrman et al. 1992; Foltin & Haney 2000). Evidence provided in the current study suggests that exposure to drug-paired stimuli may not only contribute to relapse, but that it may also alter the ability of the subject's immune system to deal with infection. Conditioning of the immune response is a well-documented phenomenon and can be accomplished with a wide variety of immunomodulatory stimuli, including drugs of abuse such as heroin. Considering the increased susceptibility to infection that occurs with opioid use it is possible that the conditioned alterations in certain immune parameters that occur in response to exposure to drug-paired cues might also compromise immune function and increase susceptibility to disease in recovering addicts.

There is evidence from both human and animal studies indicating that repeated exposure to drug cues without further drug use (i.e. extinction) decreases cue-induced conditioned responses and may therefore reduce drug seeking and craving (Childress et al. 1986; Calcagnetti & Schechter, 1993). Given the number of animal studies

showing decreased drug seeking following extinction this study sought to determine if it would be possible to reduce the conditioned immune response to a drug-paired environment by manipulating the animal's exposure to the conditioned cues. The data presented here indicate that both extinction and latent inhibition procedures attenuate heroin's conditioned effect on iNOS production. In the extinction experiment, rats that were repeatedly placed in the formerly drug-paired environment without drug administration did not exhibit a reduction in iNOS production when re-exposed to this environment, unlike those animals that had not undergone extinction. Some studies have shown that whereas extinction may reduce the occurrence of conditioned drug-like responses in a controlled environment such as the laboratory, these treatments may have little to no effect on rehabilitation (Dawe et al. 1993; Niaura et al. 1999). This may be due to several factors, including the ability of these cues to be easily reconditioned and the complexity of the cues themselves. Whereas the nature of the CS may be controlled in an experimental setting, there is no way to determine the exact nature of the CS that has become associated with drug delivery in a drug user. Even if all of these cues were identified, it would be extremely difficult to extinguish each one of them. In addition, some studies suggest that extinction is not a mechanism by which the subject unlearns the association between the cue and drug delivery but rather creates a new association in which the cue no longer predicts the availability of the drug (Bouton, 2004). Data also suggest that as the original association is still intact, it may easily be reconditioned with a single re-pairing of the drug and its cue (Leri and Rizos, 2005) or through a variety of other manipulations. In this case, it is likely that after repeated drug-free sessions in the formerly drug-paired environment the animal has learned that this environment no longer predicts the availability of drug. Similarly, rats who had prior exposure to the conditioning chamber did not

develop the conditioned response as readily as those animals that had no prior exposure. In this instance, the animal is less likely to develop a conditioned response to the drug-paired environment because the animal had previously learned that this environment is irrelevant. The fact that this learned irrelevance must be overcome before conditioning can occur is the basis for latent inhibition. These findings are particularly important because they validate the effects of exposure to a heroin-paired environment on iNOS induction as a true form of classical conditioning and demonstrate two methods of reducing the conditioned response.

The biological mechanisms underlying conditioned alterations in immune status are currently under investigation but several paradigms have revealed an involvement of the opioid system. For example, Lysle et al. (1992) demonstrated that the reduction in NK cell activity and lymphocyte mitogenic responses observed following exposure to cues that had previously been paired with aversive shock are mediated by the actions of endogenous opioids. Further experimentation revealed that these conditioned immune alterations were specifically mediated via centrally located mu-opioid receptors (Lylse & Perez, 1997). The conditioned effects of heroin on immune functioning may be mediated through similar mechanisms, however, these effects may also involve components of the CNS that have been implicated in addiction and conditioned responses to drug cues. Interestingly, human brain imaging data has shown changes in neural activity within specific brain regions following exposure to drug cues in former drug users (Sell et al. 2000). In addition, research has demonstrated an increase in c-Fos expression in the lateral habenula, basolateral amygdala complex, prelimbic cortex, and nucleus accumbens core in rats exposed to drug-associated CS (Miller & Marshall 2005; Zhang et al. 2005). Animal research employing pharmacological manipulations further supports the contribution of these

areas to cue-induced drug seeking behavior (See 2005; Rizos et al. 2005; Fuchs et al. 2005). Given that these brain areas appear to play a role in the acquisition and expression of conditioned responses to drug cues it is possible that the conditioned effects of heroin on immune status may also be mediated through activation of some of the same neural circuitry. Regardless of the mechanism, it is important to take into account the profound immune alterations that occur upon exposure to drug-related stimuli and to consider how the various manipulations that reduce these effects might impact the immune system's response to disease.

CHAPTER 3

CONDITIONED EFFECTS OF HEROIN ON PROINFLAMMATORY MEDIATORS REQUIRE THE BASOLATERAL AMYGDALA

Introduction

The data presented in Chapter 2 indicated that exposure to a previously-heroin paired environment will elicit reductions in nitric oxide expression similar to what is observed with actual heroin administration. In addition, these conditioned effects are susceptible to both extinction and latent inhibition indicating that this is a true form of classical conditioning. The studies described in Chapter 3 will determine whether other proinflammatory mediators, such as the cytokines TNF-α and IL-1β, may also be conditioned to environmental stimuli associated with drug administration. In addition, the role of the basolateral amygdala in the expression of heroin's conditioned effects on these immune parameters will be examined.

Heroin addiction is a chronic, debilitating disorder marked by high incidence of relapse despite repeated rehabilitation efforts. Exposure to drug cues is one of the key contributing factors to relapse mainly because these cues induce a wide variety of complex, classically conditioned physical and behavioral responses (Unnithan et al 1992; Derbas et al 2001). There has been increasing evidence from studies conducted on human subjects showing that drug-paired stimuli can cause intense craving, feelings of being 'high', galvanic skin responses, autonomic arousal, and altered neural activity in drug users (Sideroff & Jarvik 1980; Ehrman et al. 1992; Foltin &

Haney 2000). These data suggest that exposure to drug-associated stimuli may have effects similar to administration of the drug itself. Several investigators have shown that many of the physiological and behavioral responses to opioids, such has heroin and/or morphine, may be conditioned to previously drug-paired stimuli. For example, environmental stimuli that had previously been paired with morphine administration can elicit such morphine-like effects as hyperthermia when presented in the absence of morphine (Bardo & Valone, 1994; Eikelboom & Stewart, 1979; Miksic et al., 1975; Schwarz & Cunningham, 1990; Wikler & Pescor, 1967). Similar results have been seen with the conditioning of morphine's analgesic properties such that exposure to morphine-associated stimuli induces a conditioned analgesic response in the absence of drug (Miller et al 1990; Bardo & Valone 1994). Current research in the area of drug addiction has focused on the critical task of reducing relapse by attempting to curb cue-induced drug seeking behavior, however, it is also imperative to take into account the widespread effects these cues may have on immune functioning and the ability of previous drug users to combat infectious disease while in recovery.

Research in our laboratory and others has shown that not only will administration of heroin suppress a number of basic immune parameters but many of these suppressive effects on the immune system may also be conditioned to environmental cues that predict drug availability. For instance, rats re-exposed to a previously morphine–paired environment display alterations in basic immune parameters similar to those seen upon actual morphine administration including decreased mitogen responsiveness of lymphocytes, decreased NK cell activity and suppressed IL-2 production (Coussons et al 1992; Saurer et al 2008). In line with these studies, it has been shown that heroin administration results in a profound

reduction in LPS-induced nitric oxide expression (Lysle & How 2000) and this effect on nitric oxide may be conditioned to environmental stimuli that predict drug availability (Lysle & Ijames, 2002). In addition to effects on nitric oxide, there is evidence that opiate administration may also alter the production of the pro-inflammatory cytokines, TNF-α and IL-1β (Chao et al 1993; Pacifici et al 2000). All three of these molecules are important components of the innate immune system which provides the first line of defense against invading pathogens. Given the critical nature of these pro-inflammatory mediators in the initial response to infection it is imperative that we understand how heroin and heroin related cues may alter their effectiveness.

Little is known about the brain areas involved in these conditioned effects, however, the basolateral amygdala (BLA) has been implicated in the formation and expression of stimulus-reward associations within models of drug abuse. For example, exposure to drug cues will reinstate drug seeking behavior in rats who have undergone extinction training (Gracy et al., 2000). However, inactivation of the BLA will block the ability of these cues to reinstate drug seeking (McLauglin & See 2003; Fuchs et al 2005). Interestingly, this same study did not show an effect of BLA inactivation on drug self-administration suggesting that the BLA is critical for conditioned but not unconditioned reinforcement (Meil & See, 1997). Furthermore, animals receiving morphine injections paired with a distinct environment showed greater Fos activation within the BLA when re-exposed to that environment than animals receiving un-paired injections (Harris & Ashton-Jones, 2003). Similarly, cocaine-associated cues elicited neural activity within the BLA nearly identical to that seen upon intravenous delivery of cocaine in rats taught to self-administer (Carelli et al., 2003). Taken together, these studies provide clear and consistent evidence supporting

the investigation of the BLA as part of the neurobiological circuitry involved in the conditioned immune responses to heroin.

The data presented in Chapter 2 revealed that the conditioned effects of heroin on nitric oxide production constitute a true form of classical conditioning as these effects are susceptible to both extinction and latent inhibition. The current set of experiments sought to establish the role of the basolateral amygdala, an area known to be involved in associative learning processes, in the conditioned effects of heroin on nitric oxide by quantifying iNOS expression in spleen and liver tissue following inactivation of the BLA. In addition, IL-1β and TNF-α were measured in these same tissues to determine whether exposure to stimuli previously paired with heroin would alter the expression of these cytokines and if inactivation of the BLA would have an effect on these conditioned alterations.

Materials and Methods

Animals

Male Lewis rats, weighing 225-250 g, were purchased from Charles River Laboratories (Raleigh, N.C., USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12h) cycle maintained through artificial illumination. Animals were allowed access to food and water ad libitum throughout the experiment except for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the University of North Carolina at Chapel Hill and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD) and dissolved in 0.9 % sterile saline. For all experiments, animals received a subcutaneous injection of heroin at a dose 1 mg/kg immediately prior to placement in the conditioning chamber on each of the five conditioning trial days. This dose was selected based on prior experiments in our laboratory showing that a 1 mg/kg dose of heroin alters LPS-induced iNOS mRNA expression in spleen and liver tissue and induces conditioning (Lysle & How, 2000; Lysle & Ijames, 2002; Szczytkowski & Lysle, 2007).

Surgeries and microinjections

Animals were anesthetized with 0.35 ml intramuscular injections of 1:1 (vol/vol) ketamine hydrochloride (100 mg/ml) mixed with xylazine (20 mg/ml) and placed into the stereotaxic apparatus. Animals were implanted with 26-gauge bilateral guide cannula (Plastics One, Roanoke, VA) directed towards the BLA (AP -2.5, ML±5.0, DV -6.6) or Caudate control region (AP -2.4, ML±4.5, DV -4.0). Coordinates are expressed as millimeters from bregma (Paxinos & Watson, 1986).

Animals were given a two week recovery period before the start of conditioning trials. On test day, animals received bilateral intracranial injections (0.3 µl/side infused over 1 min) of saline vehicle or a combination of GABA agonists, muscimol (0.03 nmol)/baclofen (0.3 nmol), 30 minutes prior to re-exposure to the conditioning chambers to determine whether the brain areas inactivated are necessary for the conditioned alterations in pro-inflammatory mediators. Injectors extended 2 mm beyond the tip of the cannula and were left in place for 1 minute after the injection to allow for proper infusion.

Procedures

Acquisition of conditioned response. All animals received five 60-min training sessions in which they were administered a subcutaneous injection of heroin (1 mg/kg) upon placement into a standard conditioning chamber which served as the conditioned stimulus. Training sessions were separated by 48 hours. The conditioning chambers (BRS/LVE, Laurel, Md., USA) were contained in a room separate from the animal colony. Chambers were fitted with a metal grid floor design and cedar bedding to create an environment distinct from that of the home cage and to provide both olfactory and tactile cues for conditioning. All conditioning took place during the dark phase of the light cycle and the conditioning chambers were kept dark to minimize effects on circadian rhythms.

Treatment Groups by Experiment

BLA:	Days 1-9	Test Day (D15):	BLA Microinfusion	CS Exposure
HC Saline-BLA	Conditioning		Saline	Home Cage
CS Saline-BLA	Conditioning		Saline	CS Re-Exposure
HC Mus/Bac-BLA	Conditioning		Muscimol/Baclofen	Home Cage
CS Mus/Bac-BLA	Conditioning		Muscimol/Baclofen	CS Re-Exposure

Caudate:	Days 1-9	Test Day (D15):	Caudate Microinfusion	CS Exposure
HC Saline-Caud	Conditioning		Saline	Home Cage
CS Saline-Caud	Conditioning		Saline	CS Re-Exposure
HC Mus/Bac-Caud	Conditioning		Muscimol/Baclofen	Home Cage
CS Mus/Bac-Caud	Conditioning		Muscimol/Baclofen	CS Re-Exposure

Table 3.1: Schematic representation of the behavioral and pharmacological manipulations employed for each treatment group.

Testing of expression of conditioned response. The test day took place six days following the final conditioning session. Thirty minutes prior to testing, two groups of animals (Mus/Bac-BLA; Mus/Bac-Caud) received intra-BLA (or intra-caudate)

microinfusions of the combination of GABA agonists, muscimol and baclofen, to temporarily inactivate the BLA (or caudate). Control animals received intra-BLA (or intra-caudate) microinfusions of saline vehicle (Saline-BLA; Saline-Caud). To test the expression of the conditioned response, two groups of animals were re-exposed to the conditioned stimulus (i.e.; the conditioning chambers) without drug to determine whether the conditioned stimulus alone would induce alterations in pro-inflammatory mediators. Animals re-exposed to the conditioning chambers on test day are indicated in the figures as CS. The remaining animals (home cage, HC) were returned to the home cage following microinfusions and served as controls. Experimental treatment of each group is outlined in Table 3.1. After the 60-min re-exposure, the animals were removed from the chambers and given a subcutaneous injection of LPS (1000 μg/kg) to induce iNOS production in spleen and liver tissue. Home cage animals also received LPS at this time. Six hours after LPS administration all animals were sacrificed and samples of spleen, liver and blood were collected for analysis. The 6-hr timepoint was selected based on previous research in our laboratory showing maximal iNOS induction at six hours following LPS administration (Lysle & How, 2000).

Real Time RT-PCR

To determine iNOS expression, real time RT-PCR was performed on tissue samples from the spleen and liver. A detailed description of this procedure is provided in Chapter 2.

ELISA

For IL-1β and TNF-α protein determinations, protein was extracted from a section of each tissue using freeze/thaw lysis in tris-buffer containing antiproteinases. Protein

was quantified spectrophotometrically (Bio-Tek, Model EL312 kinetic reader, Winooski, VT, USA) using Bio-Rad protein dye. The BioSource International, Inc. (Carlsbad, CA) rat IL-1β and TNF-α ELISA test kits were used to determine the levels of IL-1β or TNF-α protein in each tissue sample. Briefly, samples and standards were added to microtiter wells coated with antibody that recognizes IL-1β or TNF-α and incubated at room temperature. Wells were washed extensively and then incubated with biotinylated antibody, followed by a second wash and then incubation with Streptavidin-HRP. After the final washing, a chromagen substrate solution was added which reacted with the bound enzyme to produce color. The color intensity developed proportionally to the amount of IL-1β or TNF-α present in each sample. The enzyme reaction was stopped after 30 minutes, and the absorbance at 450 nm was measured with a Bio-Tek (Winooski, VT) Model EL312 kinetic reader. A standard curve was obtained by plotting the absorbance versus the corresponding concentrations of the supplied standards.

Nitrite/Nitrate Assay

The level of nitrite/nitrate in plasma samples was assessed using the Greiss reagent assay. A detailed description of this procedure is provided in Chapter 2.

Statistical Analysis

Analysis of variance was performed on all data sets. When the overall ANOVA showed significant effects, *post hoc* comparisons were made using Tukey's test to compare individual treatment groups. All analyses were conducted with the level of significance set at $p < 0.05$.

Histology

To confirm proper cannula placement Alcian blue dye was infused via the cannula following sacrifice. Brains were then extracted and post-fixed in a 4% paraformaldehyde solution. Following fixation the brains were transferred to a 30% sucrose solution for cryoprotection and then frozen at -80^{o} C until further analysis. Coronal sections (50 μm) were taken and stained with cresyl violet for verification of cannula placement. Animals with cannula placement outside of the targeted region were removed from the analyses. Figure 3.1 shows a schematic diagram illustrating the most ventral point of the injection cannula for each animal in the study. Figure 3.2 is a representative photomicrograph showing the injection cannula tract to the BLA.

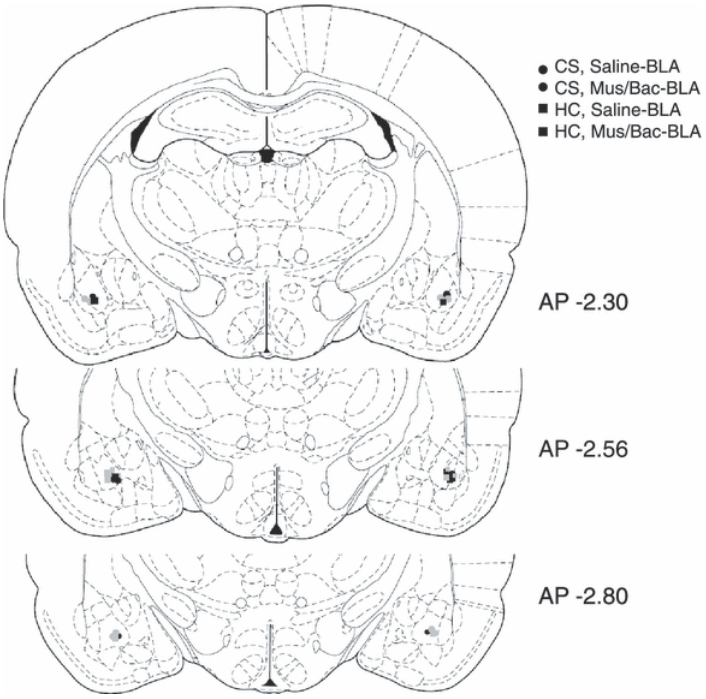

Figure 3.1: BLA injection cannula placement was verified by cresyl violet stained coronal sections. Symbols in the diagram represent the most ventral point of the injection cannula for each animal in the study based on the atlas of Paxinos and Watson (1998). AP coordinates indicate the distance from bregma in mm. (CS, Saline-black circles; CS, Mus/Back-gray circles; HC, Saline-black squares; HC, Mus/Bac-gray squares)

Figure 3.2: Representative photomicrograph showing the cannula tract and site of injection within the BLA on a cresyl violet stained coronal section. The arrow indicates the most ventral point of the injector cannula tract.

Results

Effect of BLA Inactivation on Conditioned Nitric Oxide Suppression

The first study investigated the effect of temporary inactivation of the BLA on the expression of heroin-induced conditioned suppression of nitric oxide production. Six days following the final conditioning session, animals were divided into groups with one group being re-exposed to the previously heroin-paired environment for 60 min (Conditioned Stimulus, CS) while the other group remained in the home cage and served as controls (Home Cage, HC). In order to temporarily inactivate the brain regions under investigation, animals in the Mus/Bac-BLA and Mus/Bac-Caud groups received microinfusions of the muscimol/baclofen combination directly into the BLA or Caudate, respectively, 30 minutes prior to testing. The animals in the Saline-BLA and Saline-Caud groups received microinfusions of saline into these same areas.

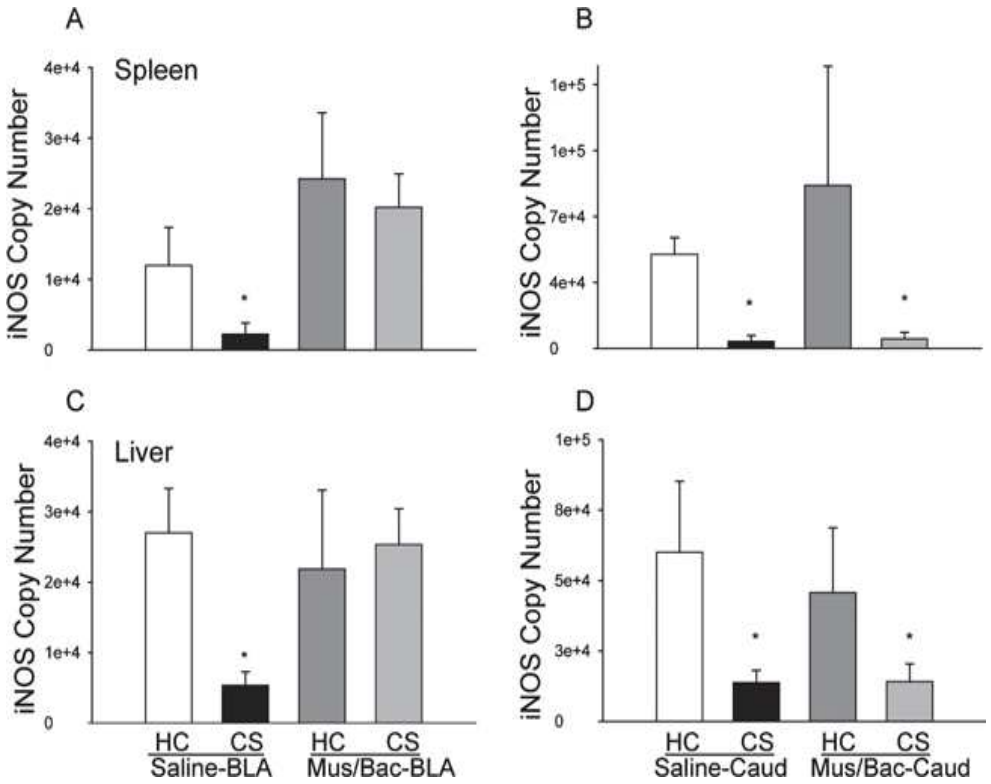

Figure 3.3: Effect of treatments on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. Data are expressed as iNOS copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. (*) Asterick indicates a significant difference ($p<0.05$) from the HC (Home cage), Saline-BLA/Caud group.

Figure 3.3 (panels A & C) shows the results of each treatment on LPS-induced expression of iNOS mRNA in the spleen and liver following inactivation of the BLA. Analysis of iNOS copy number in spleen and liver revealed a significant main effect of treatment [$F(4,15)=10.711$, $P<0.01$; $F(4,15)=9.432$, $p<0.01$]. Moreover, there was a significant difference in iNOS mRNA copy number between the animals exposed to the conditioned stimulus on test day who had received intra-BLA saline (CS, Saline-BLA) and the animals who remained in the home cage on test day and received intra-BLA saline (HC, Saline-BLA). These differences were evident in both tissues ($p<0.05$) which supports our earlier findings indicating that exposure to a previously heroin-paired environment alters the expression of iNOS mRNA. Most importantly, there were no significant differences between the HC, Saline-BLA group and the

experimental group that received intra-BLA microinjections of muscimol/baclofen before exposure to the conditioned stimulus on test day (CS, Mus/Bac-BLA) demonstrating the ability of BLA inactivation to reduce the conditioned response. Furthermore, there were no significant differences between the HC, Saline-BLA group and the group that received intra-BLA muscimol and baclofen but remained in the home cage on test day (HC, Mus/Bac-BLA) indicating that these results are specific to administration of drug and not due to ancillary effects of the microinjections. There were also no differences found in expression of the housekeeping gene, cyclophilin, between any of the groups indicating that the alterations in mRNA were specific for iNOS and not the result of an overall reduction in RNA expression or production (data not shown).

Figure 3.3 (panels B & D) also shows the effects of each treatment on LPS-induced expression of iNOS mRNA in the spleen and liver following administration of the muscimol/baclofen comebination into a control region of the caudate. Analysis of iNOS copy number in spleen and liver revealed a significant main effect of treatment [$F(4,15)=6.458$, $p<0.05$; $F(4,15)=6.868$, $p<0.01$]. Planned comparisons revealed a significant difference between the CS, Saline-Caud group and the HC, Saline-Caud group in both tissues ($p<0.05$) which is in agreement with the data from our previous research. Most importantly, there was also found to be a significant difference between the HC, Saline-Caud group and the experimental group that received intra-caudate microinjections of muscimol/baclofen that was re-exposed to the conditioned stimulus on test day (CS, Mus/Bac-Caud) indicating that inactivation of this region does not alter the heroin-induced conditioned reduction of iNOS ($p<0.05$). Furthermore, there were no significant differences between the HC, Saline-Caud group and the HC, Mus/Bac-Caud group indicating that these results are

specific to administration of drug and not due to ancillary effects of the microinjections.

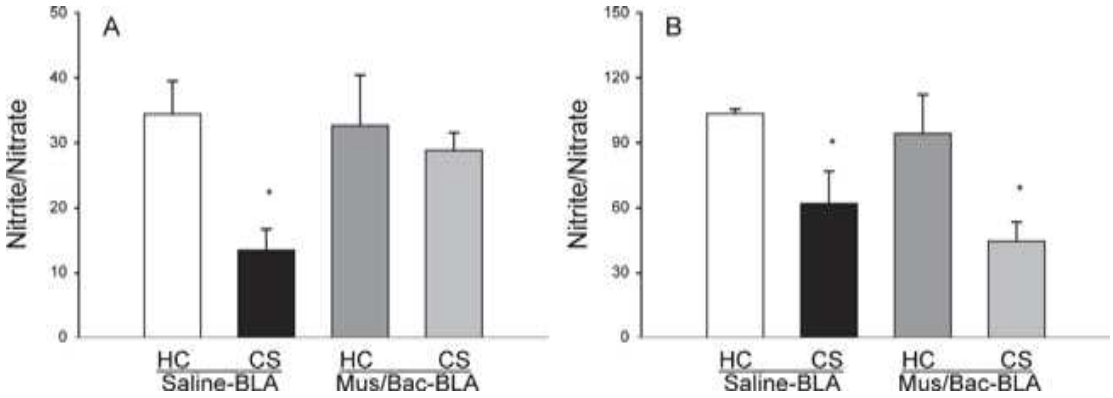

Figure 3.4: Effect of treatments on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the micromolar concentration of nitrite/nitrate. (*) Asterick indicates a significant difference ($p<0.05$) from the HC (Home cage), Saline-BLA/Caud group.

The data in Figure 3.4 show the effects of BLA (A) or caudate control region (B) inactivation on serum nitrite/nitrate levels. Nitrite and nitrate are byproducts of nitric oxide production and levels of these markers serve as an indirect analysis of nitric oxide levels in the blood. For the BLA inactivation experiment, examination of nitrite/nitrate levels in the serum revealed a significant main effect of treatment [$F(4,15)=13.939$, $p<0.01$] and a significant difference between the CS, Saline-BLA group and the HC, Saline-BLA group ($p<0.05$) indicating that exposure to a previously heroin paired environment reduces the production of nitric oxide. There were no significant differences between the HC, Saline-BLA group and the CS, Mus/Bac-BLA group demonstrating that inactivation of the BLA reduces the conditioned effects of heroin on nitric oxide. Taken together, these findings show that inactivation of the BLA will reduce the conditioned effects of heroin on nitric oxide production.

For the caudate control region experiment, analysis of variance revealed a significant main effect of treatment [$F(4,15)=16.767$, $p<0.01$] and a significant difference between both the CS, Saline-Caud group and the HC, Saline-Caud group ($p<0.01$) and the CS, Mus/Bac-Caud group and the HC, Saline-Caud group ($p<0.01$). These results indicate that exposure to a previously heroin-paired environment reduces the production of nitric oxide and this effect is not attenuated by inactivation of this region of the caudate.

BLA inactivation and the conditioned suppression of pro-inflammatory cytokines

Figure 3.5 shows that the suppressive effect of heroin on the pro-inflammatory cytokine, IL-1β, can be conditioned to environmental stimuli and this conditioned effect is reduced by inactivation of the BLA. This effect was evident at both the protein and mRNA level and in both spleen and liver tissue. Analysis revealed a main effect of treatment on both mRNA and protein levels in the spleen [$F(4,15)=6.546$, $p<0.01$; $F(4,15)=38.871$, $p<0.01$] and liver [$F(4,15)=7.993$, $p<0.01$; $F(4,15)=21.964$, $p<0.01$]. In line with our previous experiments, the CS, Saline-BLA group exhibited significantly lower levels of IL-1β mRNA and protein in both the spleen and liver ($p<0.05$) as compared to the HC, Saline-BLA group. The CS, Mus/Bac-BLA group was not significantly different from the HC, Saline-BLA group indicating that inactivation of the BLA was able to reverse the conditioned effects of heroin on IL-1β mRNA and protein expression.

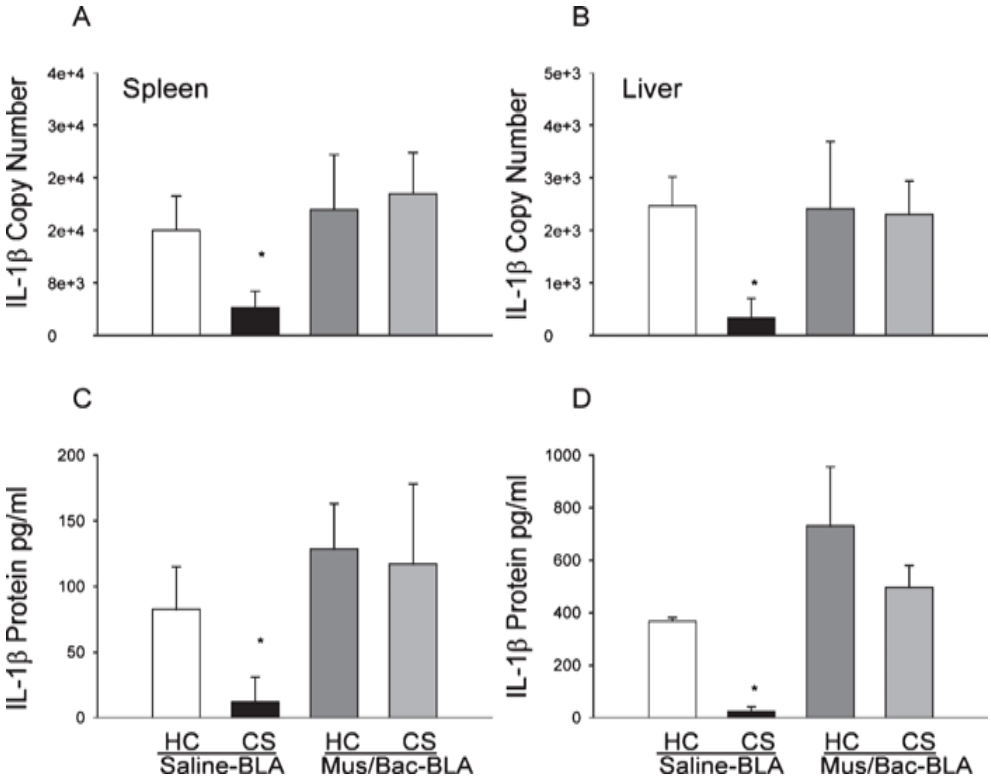

Figure 3.5: Effect of treatments on LPS-induced expression of IL-1β mRNA and protein in the spleen (A, C) and liver (B, D) as determined by real-time RT-PCR. The RT-PCR data (A, B) are expressed as IL-1β copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. The ELISA data (C, D) are expressed as picograms of protein per ml. (*) Asterick indicates a significant difference ($p<0.05$) from the HC (Home cage), Saline-BLA group.

Figure 3.6 shows that the suppressive effect of heroin on the proinflammatory cytokine IL-1β is not altered by inactivation of the caudate control region. As the time of tissue collection was optimized for iNOS analysis it is not surprising that there was no significant effect at this timepoint in liver IL-1β mRNA levels. As the effect is evident at the protein level this suggests that it might also have been present at the mRNA level at an earlier timepoint. Analysis revealed a main effect of treatment on both mRNA and protein levels in the spleen [$F(4,15) = 20.141$, $P < 0.01$; $F(4,15) = 21.585$, $P < 0.01$] and on protein in the liver [$F(4,15) = 7.993$, $P < 0.01$]. In line with our previous experiments, the CS Saline-Caud group exhibited significantly lower levels of IL-1β mRNA and protein in the spleen and in protein in the liver

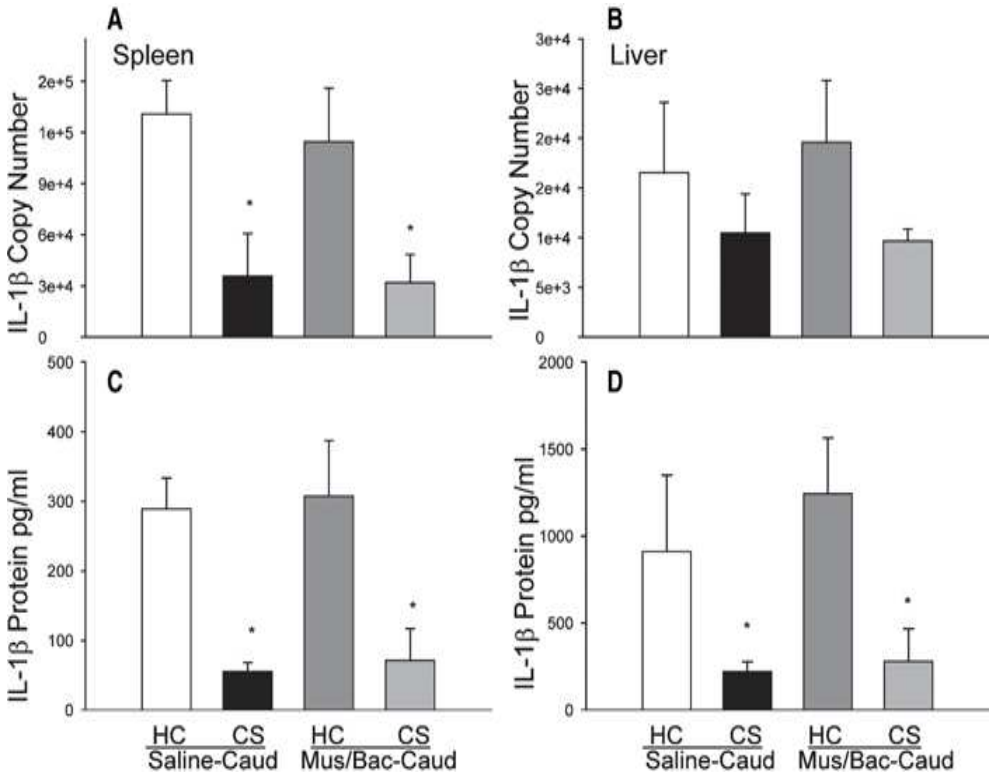

Figure 3.6 Effect of treatments on LPS-induced expression of IL-1β mRNA and protein in (A and C) the spleen and (B and D) liver as determined by real-time RT-PCR. Re-exposure to the previously heroin-paired environment (CS) reduced IL-1β mRNA and protein in the spleen and IL-1β protein in liver tissue, and this reduction was not altered by inactivation of the caudate control region. (A and B) The RT-PCR data are expressed as IL-1β copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. (C and D) The ELISA data are expressed as pg of protein per ml. *$P < 0.05$ vs. the HC Saline-Caud group.

($P < 0.05$) as compared to the HC Saline-Caud group. The CS Mus/Bac-BLA group also exhibited significantly lower IL-1β protein and mRNA (except for liver mRNA which showed the same trend but was not significant) compared with the HC Saline-Caud group, indicating that inactivation of the caudate control region was not able to reverse the conditioned effects of heroin on IL-1β mRNA and protein expression.

Figure 3.7 shows that the suppressive effect of heroin on the pro-inflammatory cytokine, TNF-α, can also be conditioned to environmental stimuli and, again, this conditioned effect is reduced by inactivation of the BLA. This effect was evident at both the protein and mRNA level in the spleen and liver with the exception of TNF-α

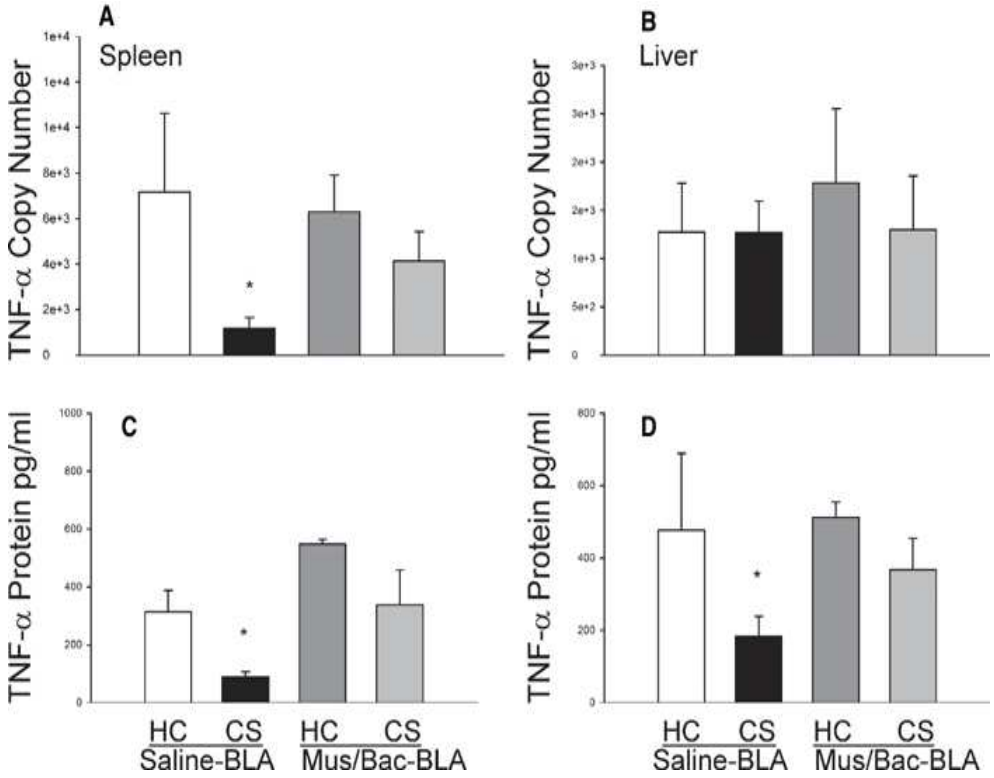

Figure 3.7 Effect of treatments on LPS-induced expression of TNF-α mRNA and protein in (A and C) the spleen and (B and D) the liver as determined by real-time RT-PCR. The RT-PCR data are expressed as TNF-α copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. (C and D) The ELISA data are expressed as pg of protein per mL. *P < 0.05 vs. the HC Saline-Caud group.

mRNA in the liver. As with IL-1β, the lack of a significant effect in liver TNF-α mRNA is not surprising and the significance at the protein level suggests that an effect may have been evident at an earlier timepoint. Analysis revealed a main effect of treatment on both mRNA and protein levels in the spleen [$F(4,15)=6.946$, $p<0.01$; $F(4,15)=27.644$, $p<0.01$] and on protein levels in the liver [$F(4,15)=7.313$, $p<0.01$]. In line with our previous experiments, the CS, Saline-BLA group exhibited significantly lower levels of TNF-α mRNA in the spleen ($p<0.05$) and significantly lower levels of TNF-α protein in both the spleen and liver ($p<0.05$) when compared to the HC, Saline-BLA group.

Figure 3.8 shows that the suppressive effect of heroin on the pro-inflammatory cytokine, TNF-α, is not altered by inactivation of the caudate control region. The

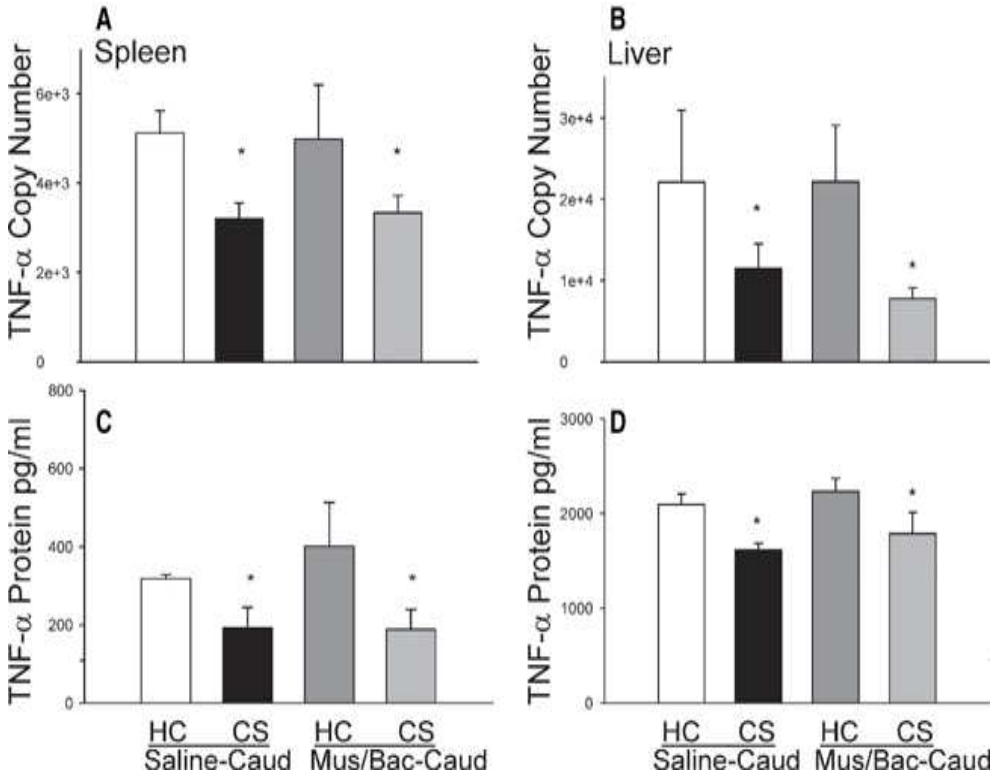

Figure 3.8: Effect of treatments on LPS-induced expression of TNF-α mRNA and protein in the spleen (A, C) and liver (B, D) as determined by real-time RT-PCR. The RT-PCR data (A, B) are expressed as TNF-α copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software. The ELISA data (C, D) are expressed as picograms of protein per ml. (*) Asterick indicates a significant difference ($p<0.05$) from the HC (Home cage), Saline-Caud group.

suppressive effect of conditioning was evident at both the protein and mRNA level. Analysis revealed a main effect of treatment on both mRNA and protein levels in the spleen [$F(4,15)=7.605$, $p<0.01$.; $F(4,15)=7.779$, $p<0.01$] and liver [$F(4,15)=11.494$, $p<0.01$; $F(4,15)=12.655$, $p<0.01$]. In line with our previous experiments, the CS, Saline-Caud group exhibited significantly lower levels of TNF-α mRNA and protein in the spleen and in the liver when compared to the HC, Saline-Caud group. Inactivation of the caudate control region had no effect on this suppression as the CS, Mus/Bac-Caud group also showed significantly lower levels of TNF-α protein and mRNA as compared to the HC, Saline-Caud group ($p<0.05$).

Discussion

Previous research in our laboratory has shown that exposure to a previously drug-paired environment induces alterations in nitric oxide production similar to those observed upon actual drug administration (Lysle & Ijames, 2002; Szczytkowski & Lysle, 2007). The data presented here are the first to demonstrate that the suppressive effects of heroin on the pro-inflammatory cytokines, TNF-α and IL1-β, may also be conditioned to environmental stimuli. Both Interleukin-1 beta (IL-1β) and Tumor necrosis factor-alpha (TNF-α) are pro-inflammatory cytokines involved in host defense. When secreted, both IL-1β and TNF-α have widespread effects in the host including altering the thermoregulatory setting in the hypothalamus to produce fever and increasing the expression of adhesion factors on endothelial cells to promote the transmigration of leukocytes to sites of infection. In addition, it appears that both TNF-α and IL-1β are highly involved in LPS-induced septic shock. Rats with LPS-induced septic shock exhibited a peak in TNF-α mRNA in plasma at one hour post-infection with levels beginning to decline by two hours after LPS administration. Similarly, IL-1β peaked at one hour post-LPS and gradually returned to baseline over nine hours (Lin et al, 2006). The samples collected in this study were taken at 6 hours post-LPS injection to optimize for LPS-induced nitric oxide production. The ability of these manipulations to detect a significant change in IL-1β and TNF-α demonstrates the robustness of these effects. Furthermore, given the time course of these effects the fact that some of the data failed to reveal significant differences between treatment groups in levels of TNF-α or IL-1β mRNA in the liver is not surprising. However, the significant reduction in TNF-α and IL-1β protein in liver tissue detected by the ELISA suggests that mRNA levels for these cytokines may

have been previously suppressed in the group that was re-exposed to the conditioned stimulus on test day but may have rebounded by the 6-hour timepoint at which they were analyzed.

Given the multifaceted and complex involvement of these cytokines, as well as nitric oxide, in the initial immune response to infectious challenge it is essential to understand the mechanisms and neural circuitry through which heroin's conditioned alterations of these pro-inflammatory mediators are controlled. The results presented here provide the first evidence that inactivation of the BLA can reduce or even eliminate the conditioned suppressive effects of heroin on nitric oxide induction and on the expression of the pro-inflammatory cytokines, TNF-α and IL-1β. This attenuation appears to encompass several organ systems as it is found in both spleen and liver tissues as well as in serum levels of nitrite/nitrate. In addition, the attenuation is apparent at both the mRNA and protein levels showing that the expression as well as the production of these mediators is altered. The use of several control groups further demonstrates that these results are specific to the manipulations used and not related to ancillary effects of the conditioning or surgical procedures. Moreover, in order to ensure that these results were specific to the BLA, a control experiment was conducted in which an area of the caudate situated dorsal to the BLA was also injected with the combination of GABA agonists. The inactivation of this region of the caudate did not produce an attenuation of the conditioned response demonstrating that the results are specific to the BLA. These findings are consistent with prior investigations showing that heroin-induced alterations in immune status may be conditioned to drug-paired stimuli (Lysle & Ijames, 2002) and that these alterations are indeed a form of associative learning as they are susceptible to both extinction and latent inhibition (Szczytkowski & Lysle, 2007). In these previous

investigations, animals received subcutaneous injections of heroin upon placement in a distinctive environment. Upon re-exposure to that environment, in the absence of drug, the subjects exhibited a reduction in nitric oxide production similar to what is observed with heroin treatment. Repeated exposure to the distinctive environment without drug, either pre- (latent inhibition) or post-conditioning (extinction) abolished this conditioned effect. The present study extends these previous findings by showing that the basolateral amygdala, an area known to be involved in associative learning processes, is required for the expression of these conditioned effects.

These results suggest that there exists a neural circuit, of which the BLA is an integral component, which mediates these conditioned alterations in immune measures. Given the wealth of research showing the involvement of the BLA in conditioned responses, it is not surprising that this area would be required for the expression of conditioned suppression of pro-inflammatory mediators. Numerous investigators have demonstrated a role of the BLA in associative learning. For example, lesions of the BLA block acquisition of classical eye blink conditioning (Blankenship et al., 2005), inhibit conditioned fear responses to aversive stimuli in rats (Lee et al., 2005), and block acquisition of the motivational value of an appetitive US (Setlow et al., 2002). In addition, learning of an inhibitory avoidance task involves neuronal activity within the BLA (Chang et al., 2005) as does olfactory fear conditioning (Sevelinges et al., 2004) and conditioning of odorant attractiveness in female mice (Moncho-Bogani et al., 2005). Furthermore, the BLA has been implicated in the formation of new, and in the utilization of already established, stimulus-reward associations within models of drug abuse. For example, inactivation of the BLA inhibits the reacquisition of heroin seeking in a test of conditioned place preference (Rizos et al., 2005). Similarly, it has been demonstrated that inactivation

of the BLA abolishes the expression of CS-induced reinstatement of heroin-seeking behavior in rats (Fuchs & See, 2002). In addition, it has been shown that animals receiving morphine paired with a distinct environment exhibit Fos protein expression, a marker of neural activation, within both the BLA and the central nucleus of the amygdala during a test of conditioned place preference (Harris & Ashton-Jones, 2003).

The exact mechanism through which conditioned drug cues exert their effects on pro-inflammatory mediators remains unknown as does the means by which the BLA alters these effects. Studies suggest that it is unlikely the BLA itself has a direct effect on immune parameters as lesions and/or inactivation of this area do not seem to induce changes in immune reactivity (Jurkowski et al 2001; Grijalva et al 1990). This is consistent with the data reported here since inactivation of the BLA, by itself, did not alter the induction of pro-inflammatory mediators. While the BLA may not directly alter immune parameters, previous work in our laboratory and others has shown several brain areas to which the BLA is connected that have direct effects on the immune system including the central amygdala and the nucleus accumbens. For instance, Hayley et al (2002) reported increased monoamine utilization in the central amygdala in response to systemic administration of TNF-*a* suggesting that this area of the amygdala is sensitive to peripheral changes in cytokine production. In addition, research has shown that systemic LPS administration induces c-fos expression in the central amygdala (Rivest & LaFlamme, 1995; Sagar et al 1995; Tkacs & Li, 1999) and infusion of dopamine D1 agonists directly into this area results in increased Con-A stimulated splenocyte proliferation (Nistico et al 1994; Caroleo et al 1998). The BLA sends extensive projections to the central amygdala and the communication between these two areas is thought to create a circuit by which many conditioned

responses are mediated (Pare et al 1995; Royer et al 1999; Likhtik et al 2008). Likewise, studies have shown that interactions between the BLA and the nucleus accumbens may be necessary for the establishment and expression of some conditioned responses including cocaine seeking induced by conditioned drug cues (Setlow et al, 2002; Di Ciano & Everitt, 2004). In addition, infusion of dopamine D1 antagonists into the nucleus accumbens shell blocks morphine-induced suppression of NK cell cytotoxicity while infusion of a D1 agonist recreates this suppression (Saurer et al 2006). These data suggest that dopamine activity within the nucleus accumbens is both necessary and sufficient for the induction of immune alterations. Since inactivation of the BLA attenuates the conditioned effects of heroin on pro-inflammatory mediators it is possible that information regarding the conditioned stimulus is routed through the BLA to the nucleus accumbens and it is actually the nucleus accumbens that directly influences immune alterations.

Considering the increased susceptibility to infection that occurs with opioid use it is possible that the conditioned alterations in certain immune parameters that occur in response to exposure to drug-paired cues might also compromise immune function and increase susceptibility to disease. The host's initial response to an immune challenge is a complex and highly regulated process. Some of the key mediators involved in the initial response to potential pathogens are inducible nitric oxide, IL-1β, and TNF-α. Each of these immune system components is responsible for numerous processes that together make up the response that is necessary to combat infectious agents and to prepare the site of infection for repair and recovery. These results are important, not only because they provide the first demonstration that the suppression of pro-inflammatory cytokines can be conditioned to environmental stimuli paired with heroin administration, but also because they demonstrate that the

expression of the conditioned effects of heroin on pro-inflammatory mediators require the basolateral amygdala. These effects and the brain areas that control them need to be factored into the comprehensive treatment of opiate users.

CHAPTER 4

THE ROLE OF DOPAMINE IN HEROIN-INDUCED CONDITIONED IMMUNOMODULATION

Introduction

The results presented in chapters 2 and 3 indicated that exposure to environmental stimuli previously paired with heroin administration will elicit alterations in immune paramaters and these conditioned effects are attenuated by inhibition of the basolateral amygdala. Those findings suggest that the basolateral amygdala is an integral component of the circuit mediating the conditioned effects of heroin on proinflammatory mediators. The current chapter further explores the role of basolateral amygdala by examining the dopaminergic inputs to this brain region and their involvement in the expression of heroin-induced conditioned immunomodulation.

Interestingly, the dopaminergic system within the BLA has been shown to be involved in learning and memory and specifically the associative learning that underlies classical conditioning. For example, intra-amygdalar 6-hydroxydopamine injections impaired (Ashford & Jones, 1976), while post-training intra-BLA dopamine infusions enhanced (LaLumiere et al., 2004) memory consolidation of a conditioned avoidance task. In addition, rats with 6-hydroxydopamine lesions of the amygdala exhibit impaired acquisition of conditioning to explicit cues (Selden et al., 1991). Microdialysis studies revealed an increase in dopamine and its metabolites during the learning of a discriminative task in rats (Hori et al., 1993) further supporting the

importance of this neurotransmitter in BLA-mediated learning and memory. Furthermore, Macedo et al. (2007) demonstrated that antagonism of dopamine D_1 receptors within the BLA reduces the expression of conditioned, but not unconditioned, fear.

Dopaminergic signaling within the BLA appears to be particularly important for the associative learning processes that accompany the acquisition and expression of conditioned responses to drugs of abuse. For example, intra-BLA administration of D-amphetamine potentiates cue-induced cocaine seeking possibly by increasing monoamine tone in this region (Ledford et al., 2003). In addition, site specific adminstration of a dopamine $D_1/D_2/D_3$ antagonist into the basolateral amygdala, but not the central amygdala, blocks alcohol-induced conditioned place preference in mice (Gremel & Cunningham, 2008). More specifically, stimulation of dopamine D_1 receptors in the BLA was found to be necessary for the expression of cue-induced reinstatement of cocaine-seeking behavior in rats (See et al., 2001; Yun & Fields, 2003). In contrast, dopamine D_2 receptors in the BLA appear to be involved in the acquisition of associations between drugs of abuse and the conditioned cues that guide subsequent cue-induced drug-seeking behavior (Berglind et al., 2006). These findings provide a strong rationale for examining the involvement of the dopaminergic system within the BLA on the expression of heroin-conditioned immunomodulation. In addition, dopamine has been implicated in the immunosuppression caused by morphine administration. Saurer et al. (2004) demonstrated that the dopamine D_1 receptor antagonist SCH23390 administered into the accumbens shell attenuates morphine-induced suppression of NK cell activity.

The current set of experiments sought to establish the role of the dopaminergic system within the basolateral amygdala in the conditioned effects of heroin on nitric

oxide by quantifying iNOS expression in spleen and liver tissue following antagonism of dopamine (D_1 or D_2) receptors in the BLA. In addition, IL-1β and TNF-α were measured in these same tissues to determine whether exposure to stimuli previously paired with heroin would alter the expression of these cytokines and if dopamine antagonism would have an effect on these conditioned alterations. The findings reported here are important because they indicate that previously heroin-associated environmental stimuli are not only capable of inducing alterations in proinflammatory mediators but that these effects may be modified by antagonism of specific dopaminergic receptors within the BLA.

Materials and Methods

Animals

Male Lewis rats, weighing 225-250 g, were purchased from Charles River Laboratories (Raleigh, N.C., USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12h) cycle maintained through artificial illumination. Animals were allowed access to food and water ad libitum throughout the experiment except for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the University of North Carolina at Chapel Hill and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD) and dissolved in 0.9 % sterile saline. For all experiments, animals received a subcutaneous injection of heroin at a dose 1 mg/kg immediately prior to placement in the conditioning chamber on each of the five conditioning trial days. This dose was selected based on prior experiments in our laboratory showing that a 1 mg/kg dose of heroin alters LPS-induced iNOS mRNA expression in spleen and liver tissue and induces conditioning (Lysle & How, 2000; Lysle & Ijames, 2002; Szczytkowski & Lysle, 2007).

Surgeries and microinjections

Animals were anesthetized with 0.35 ml intramuscular injections of 1:1 (vol/vol) ketamine hydrochloride (100 mg/ml) mixed with xylazine (20 mg/ml) and placed into the stereotaxic apparatus. Animals were implanted with 26-gauge bilateral guide cannula (Plastics One, Roanoke, VA) directed towards the BLA (AP -2.5, ML±5.0, DV -6.6). Coordinates are expressed as millimeters from bregma (Paxinos & Watson, 1986).

Animals were given a two week recovery period before the start of conditioning trials. On test day, animals received bilateral intracranial injections (0.5 μl/side infused over 1 min) of saline vehicle, the D1 antagonist, SCH23390 (2 μg/0.5 μl/side) or the D2 antagonist, raclopride (5 μg/0.5 μl/side) 30 minutes prior to re-exposure to the conditioning chambers. These doses were chosen based upon previous behavioral studies that had shown effects upon administration of these doses into the BLA (Berglind et al, 2005; See et al, 2001). Injectors extended 2 mm beyond the tip of the cannula and were left in place for 1 minute after the injection to allow for proper infusion.

Histology

To confirm proper cannula placement Alcian blue dye was infused via the cannula following sacrifice. Brains were then extracted and post-fixed in a 4% paraformaldehyde solution. Following fixation the brains were transferred to a 30% sucrose solution for cryoprotection and then frozen at -80^{o} C until further analysis. Coronal sections (50 μm) were taken and stained with cresyl violet for verification of cannula placement. Animals with cannula placement outside of the targeted region were removed from the analyses.

Procedures

Acquisition of conditioned response. All animals received five 60-min training sessions in which they were administered a subcutaneous injection of heroin (1 mg/kg) upon placement into a standard conditioning chamber which served as the conditioned stimulus. A detailed description of these training sessions is provided in Chapters 2 and 3.

Testing of expression of conditioned response. The test day took place six days following the final conditioning session. To test the expression of the conditioned response, animals were re-exposed to the conditioned stimulus (i.e.; the conditioning chambers) without drug to determine whether the conditioned stimulus alone would induce alterations in pro-inflammatory mediators. Thirty minutes prior to testing, animals received intra-BLA microinfusions of either the D1 antagonist, SCH23390, or the D2 antagonist, raclopride. Control animals received intra-BLA microinfusions of saline vehicle. Two groups of animals were then re-exposed to the chambers (conditioned stimulus, CS) for 60-min without further administration of

heroin. The remaining animals (home cage, HC) were returned to the home cage following microinfusions and served as controls. After the 60-min re-exposure, the animals were removed from the chambers and given a subcutaneous injection of LPS (1000 μg/kg) to induce iNOS, TNF-α and IL-1β production. Home cage animals also received LPS at this time. Six hours after LPS administration all animals were sacrificed and samples of spleen, liver and blood were collected for analysis. The 6-hr timepoint was selected based on previous research in our laboratory showing maximal iNOS induction at six hours following LPS administration (Lysle & How, 2000).

Real Time RT-PCR

To determine iNOS expression, real time RT-PCR was performed on tissue samples from the spleen and liver. A detailed description of this procedure is provided in Chapter 2.

ELISA

For IL-1β and TNF-α protein determinations, protein was extracted from a section of each tissue using freeze/thaw lysis in tris-buffer containing antiproteinases. A detailed description of this procedure is provided in Chapter 3.

Nitrite/Nitrate Assay

The level of nitrite/nitrate in plasma samples was assessed using the Greiss reagent assay. A detailed description of this procedure is provided in Chapter 2.

Statistical Analysis

Analysis of variance was performed on all data sets. When the overall ANOVA showed significant effects, *post hoc* comparisons were made using Tukey's test to compare individual treatment groups. All analyses were conducted with the level of significance set at $p < 0.05$.

Results

D_1 Receptor Antagonism

The first study investigated the effect of intra-BLA microinfusion of the D_1 receptor antagonist, SCH23390, on the expression of heroin-induced conditioned suppression of proinflammatory mediators. Six days following the final conditioning session, animals were divided into groups with one group being re-exposed to the previously heroin-paired environment for 60 min (Conditioned) while the control groups remained in the home cage. In order to temporarily block the receptors under investigation, animals in the SCH23390 groups received microinfusions of the SCH23390 compound directly into the BLA 30 minutes prior to testing of the conditioned response. The animals in the saline-treated groups received microinfusions of saline into these same areas.

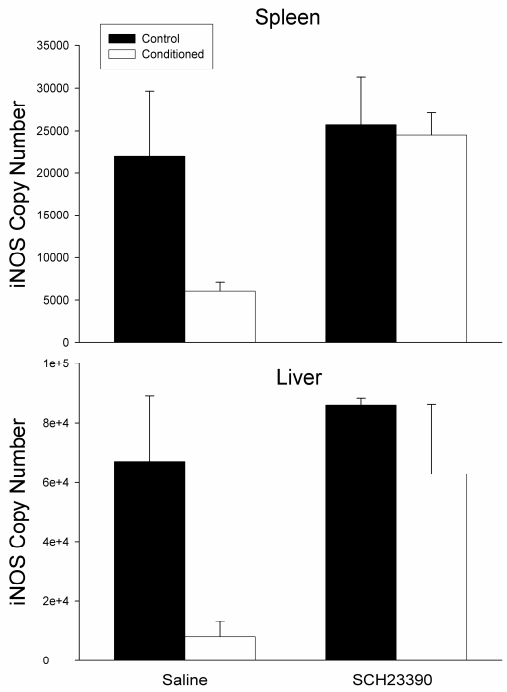

Figure 4.1: Effect of treatments on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. Data are expressed as iNOS copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

Figure 4.1 shows the mean levels of LPS-induced iNOS mRNA expresssion in the spleen and liver for each group of animals. Analysis of iNOS copy number in spleen and liver, respectively, revealed a significant main effect of group [$F(4,15)=13.716$, $P<0.01$; $F(4,15)=16.883$, $p<0.01$]. Moreover, post-hoc analyses revealed a significant difference in iNOS mRNA copy number between the saline-treated animals exposed to the conditioned stimulus on test day (Conditioned, white bars) and the animals who remained in the home cage on test day (Control, black bars). These differences were evident in both tissues ($p<0.05$) which reconfirms our earlier findings indicating that exposure to a previously heroin-paired environment suppresses the expression of iNOS mRNA. Most importantly, there were no significant differences between the saline-treated control group (Saline, black bars) and the SCH-treated conditioned group (SCH23390, black bars) for either tissue.

These results demonstrate that antagonism of dopamine D_1 receptors in the BLA blocks the conditioned effects of a previously heroin-paired environment on iNOS mRNA expression.

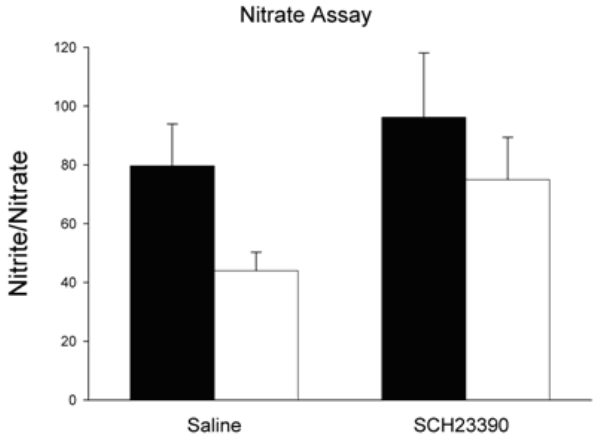

Figure 4.2: Effect of treatments on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the micromolar concentration of nitrite/nitrate.

The data in Figure 4.2 show levels of serum nitrite/nitrate levels for each group. The ANOVA revealed a significant main effect of procedure [$F(4,15) = 9.416$, $P<0.01$] on the levels of nitrite/nitrate in the serum. Post-hoc analyses revealed a significant difference between the saline-treated animals exposed to the conditioned stimulus on test day (Conditioned, white bars) and the animals who remained in the home cage on test day (Control, black bars) ($p<0.05$). These results further support our earlier findings that exposure to a previously heroin-paired environment decreases serum nitrite/nitrate levels, indicating a reduction in nitric oxide production. There were no significant differences between the SCH23390-treated group exposed to the conditioned stimulus on test day and the saline-treated control group indicating that the antagonism of dopamine D_1 receptors in the BLA attenuated the conditioned response. There were also no differences between the saline-treated control group and

the SCH23390-treated control group indicating that administration of SCH23390 alone does not alter nitrate levels in the serum.

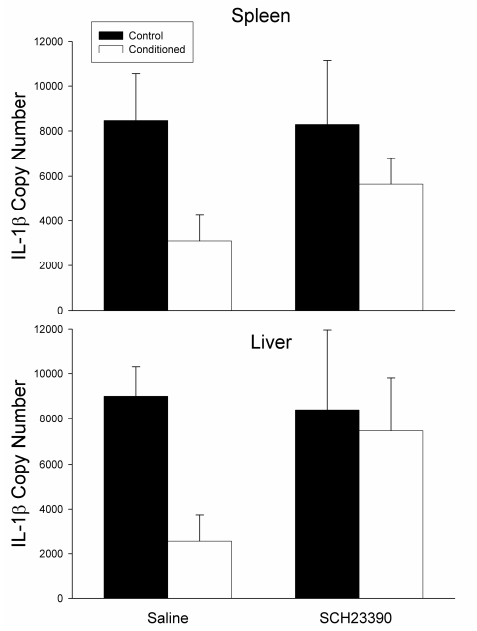

Figure 4.3 Effect of treatments on LPS-induced expression of IL-1β mRNA in the spleen and liver as determined by real-time RT-PCR. Data are expressed as IL-1β copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

Figure 4.3 illustrates that the suppressive effect of heroin on mRNA levels of the pro-inflammatory cytokine, IL-1β, can be conditioned to environmental stimuli and this conditioned effect may be reduced by antagonism of dopamine D_1 receptors within the BLA. Analysis revealed a significant main effect of treatment on mRNA levels in the spleen [$F(4,15)=6.794$, $p<0.01$] and liver [$F(4,15)=8.929$, $p<0.01$]. In line with our previous experiments, the saline-treated conditioned group exhibited significantly lower levels of IL-1β mRNA in both the spleen and liver ($p<0.05$) as compared to the saline-treated control group. Most importantly, the SCH-treated conditioned group was not significantly different from the saline-treated control group for either tissue indicating that inactivation of the BLA was able to attenuate the

conditioned effects of heroin on IL-1β mRNA. There were also no significant differences between the SCH-treated control group and the saline-treated control group indicating that SCH-treatment alone did not have an effect on IL-1β mRNA levels in the spleen or liver.

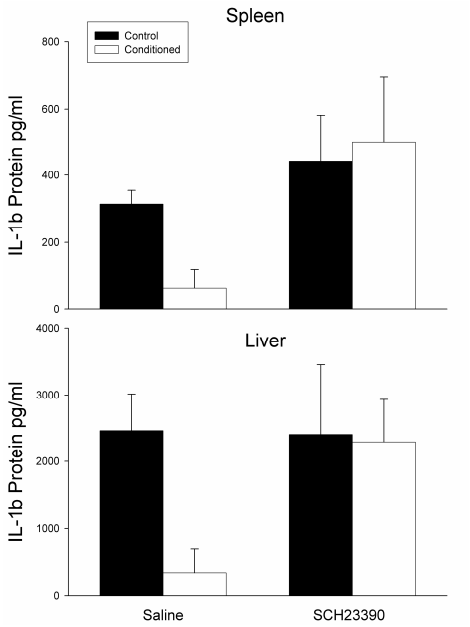

Figure 4.4 Effect of treatments on LPS-induced expression of IL-1β protein in the spleen and liver as determined by real-time RT-PCR. Data are expressed as pg of protein per ml.

Figure 4.4 shows that the results for IL-1β protein levels in spleen and liver tissue are similar to those seen for IL-1β mRNA levels. Analysis revealed a main effect of treatment on protein levels in the spleen [$F(4,15)=9.675$, $p<0.01$] and liver [$F(4,15)= 3.567$, $p<0.05$]. As expected, the saline-treated conditioned group exhibited significantly lower levels of IL-1β protein in both the spleen and liver ($p<0.05$) as compared to the saline-treated control group. In addition, the SCH-treated conditioned group was not significantly different from the saline-treated control group for either tissue similar to the results seen with IL-1β mRNA levels. There were also

no significant differences between the SCH-treated control group and the saline-treated control group.

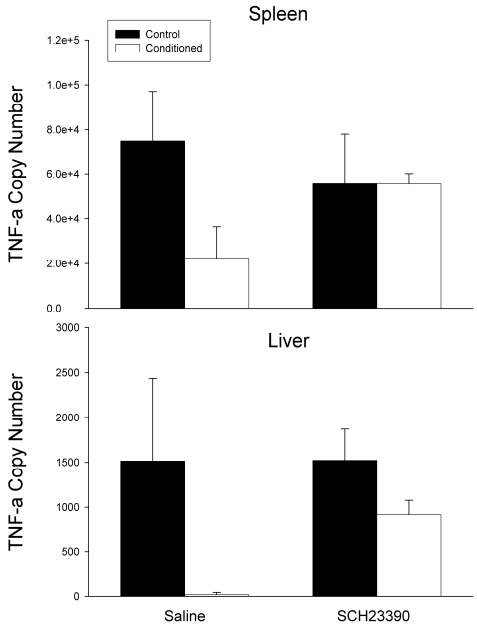

Figure 4.5: Effect of treatments on LPS-induced expression of TNF-α mRNA and protein in the spleen and liver as determined by real-time RT-PCR. The data are expressed as TNF-α copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

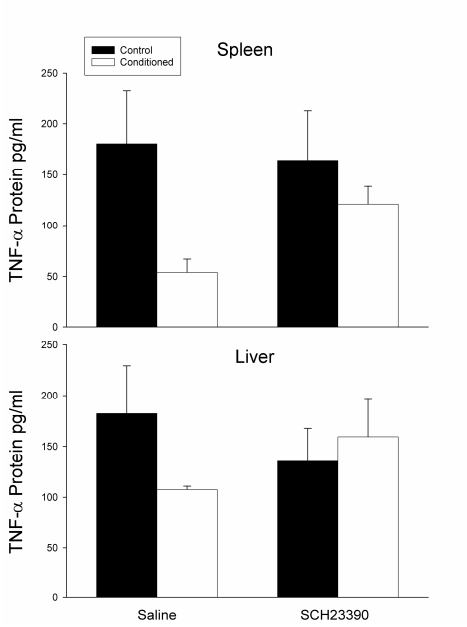

Figure 4.6: Effect of treatments on LPS-induced expression of TNF-α protein in the spleen and liver as determined by ELISA. The data are expressed as pg of protein per mL.

Figures 4.5 and 4.6 shows that the suppressive effect of heroin on the proinflammatory cytokine, TNF-α, may be conditioned to environmental stimuli and, again, this conditioned effect is reduced by antagonism of dopamine D_1 receptors within the BLA. This effect was evident at the mRNA and protein level in both spleen and liver tissue. Analysis revealed a main effect of treatment on mRNA and protein, respectively, in both spleen [$F(4,15)=6.415$, $p<0.01$; $F(4,15)=9.027$, $p<0.01$] and liver [$F(4,15)=7.313$, $p<0.01$; $F(4,15)=3.538$, $p<0.05$]. In line with our previous experiments, the saline-treated group exposed to the conditioned stimulus on test day exhibited significantly lower levels of TNF-α mRNA and protein in the spleen and liver ($p<0.05$) when compared to the saline-treated control group. In addition, there were no significant differences between the saline-treated control group and either of the SCH-treated groups.

D_2 Receptor Antagonism

The second study investigated the effect of intra-BLA microinfusion of the D_2 receptor antagonist, raclopride, on the expression of heroin-induced conditioned suppression of proinflammatory mediators. The experimental design followed that of the D_1 receptor antagonist study with one group being re-exposed to the previously heroin-paired environment for 60 min on test day while the control groups remained in the home cage. In order to temporarily block the dopamine D_2 receptors within the BLA, animals in the raclopride-treated groups received microinfusions of the D_2 antagonist directly into the BLA 30 minutes prior to testing of the conditioned response. The animals in the saline-treated groups received microinfusions of saline into the same area.

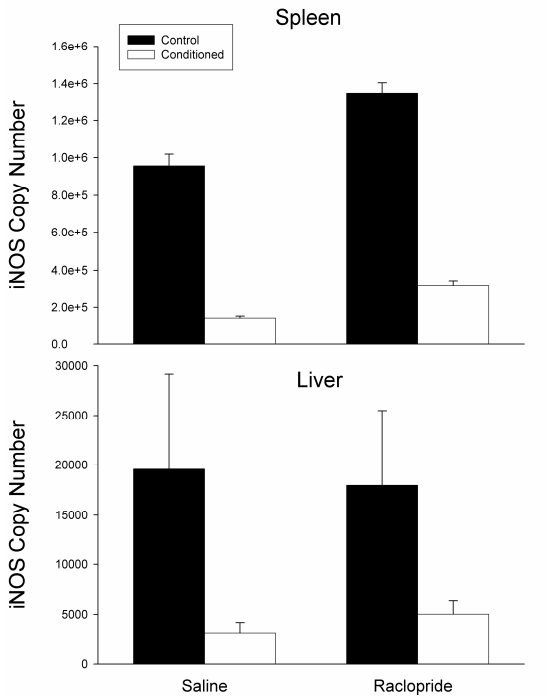

Figure 4.7: Effect of treatments on LPS-induced expression of iNOS mRNA in the spleen and liver as determined by real-time RT-PCR. Data are expressed as iNOS copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

Figure 4.7 shows the mean levels of LPS-induced iNOS mRNA expresssion in the spleen and liver for each group of animals. Analysis of iNOS copy number in spleen and liver, respectively, revealed a significant main effect of group [$F(4,15)=6.625$, $P<0.01$; $F(4,15)=7.682$, $p<0.01$]. Moreover, post-hoc analyses revealed a significant difference in iNOS mRNA copy number between the saline-treated animals exposed to the conditioned stimulus on test day (Conditioned, white bars) and the animals who remained in the home cage on test day (Control, black bars). These differences were evident in both tissues ($p<0.05$) which reconfirms our earlier findings indicating that exposure to a previously heroin-paired environment suppresses the expression of iNOS mRNA. Interestingly, there was also a significant difference between the saline-treated control group and the raclopride-treated conditioned group for both tissues. These results indicate that antagonism of

dopamine D_2 receptors in the BLA does not alter the conditioned effects of a previously heroin-paired environment on iNOS mRNA expression.

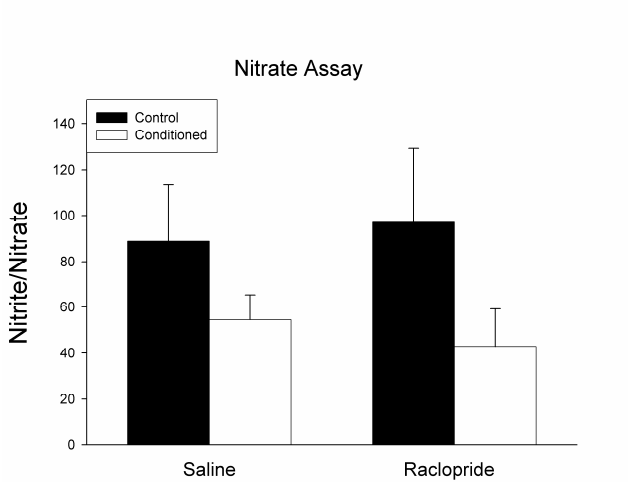

Figure 4.8: Effect of treatments on serum nitrite/nitrate levels as determined by the Greiss reagent assay. The data are expressed as the micromolar concentration of nitrite/nitrate.

The data in Figure 4.8 shows the mean levels of serum nitrite/nitrate for each experimental group. The ANOVA revealed a significant main effect of procedure [$F(4,15) = 5.442$, $P<0.05$] on serum levels of nitrite/nitrate. Post-hoc analyses revealed a significant difference between the saline-treated animals exposed to the conditioned stimulus on test day and the animals who remained in the home cage ($p<0.05$). These results are in line with our earlier experiments demonstrating that these effects may be conditioned to environmental stimuli. There was also a significant difference between the raclopride-treated group exposed to the conditioned stimulus on test day and the saline-treated control group indicating that the antagonism of dopamine D_2 receptors in the BLA does not have an effect on the conditioned response to a previously heroin-paired environment.

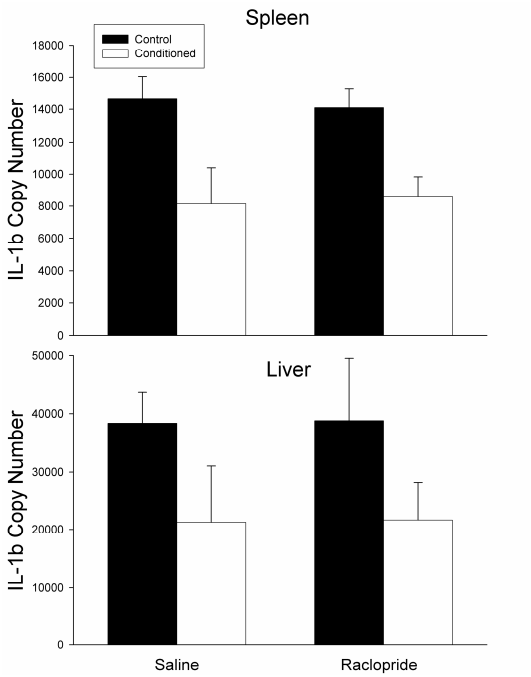

Figure 4.9 Effect of treatments on LPS-induced expression of IL-1β mRNA in the spleen and liver as determined by real-time RT-PCR. Data are expressed as IL-1β copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

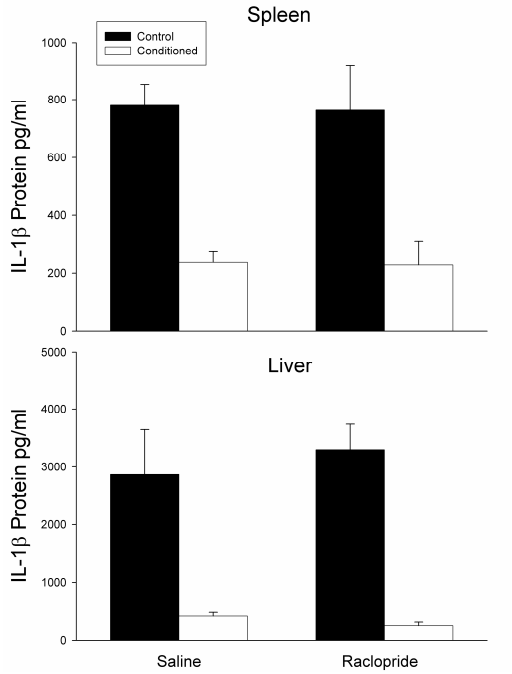

Figure 4.10 Effect of treatments on LPS-induced expression of IL-1β protein in the spleen and liver as determined by real-time RT-PCR. Data are expressed as pg of protein per ml.

Figures 4.9 and 4.10 show that the suppressive effect of heroin on mRNA and protein levels of the pro-inflammatory cytokine, IL-1β, can be conditioned to environmental stimuli and this conditioned response is not altered by antagonism of dopamine D_2 receptors within the BLA. Analysis revealed a main effect of treatment on IL-1β mRNA levels in the spleen [$F(4,15)=19.232$, $p<0.01$] and liver [$F(4,15)=5.601$, $p<0.05$] as well as protein levels in the spleen [$F(4,15)=34.440$, $p<0.01$] and liver [$F(4,15)=50.416$, $p<0.01$]. In line with our previous experiments, the saline-treated conditioned group exhibited significantly lower levels of IL-1β mRNA and protein in the spleen and liver ($p<0.05$) as compared to the saline-treated control group. Most importantly, the raclopride-treated conditioned group also displayed signicantly lower levels of IL-1β mRNA in the spleen and liver as compared to the saline-treated control group. These data suggest that antagonism of doapmine D_2 receptors within the BLA does not alter the conditioned effects of heroin on IL-1β mRNA levels. There were also no significant differences between the raclopride-treated control group and the saline-treated control group indicating that raclopride-treatment alone did not have an effect on IL-1β mRNA or protein levels in the spleen or liver.

Figures 4.11 and 4.12 show that the heroin-induced conditioned suppression of the proinflammatory cytokine, TNF-α, is not affected by antagonism of dopamine D_2 receptors within the BLA. Analysis revealed a main effect of treatment on mRNA and protein in the spleen [$F(4,15)=6.442$, $p<0.01$; $F(4,15)=5.374$, $p<0.05$] and on mRNA in the liver [$F(4,15)=18.936$, $p<0.01$]. In line with our previous experiments,

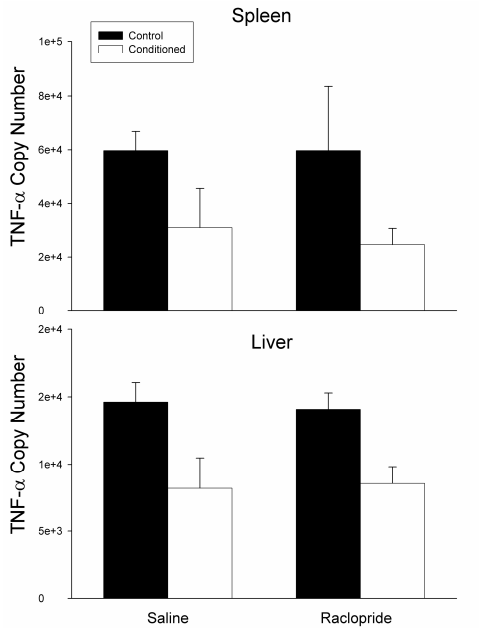

Figure 4.11 Effect of treatments on LPS-induced expression of TNF-α mRNA and protein in the spleen and liver as determined by real-time RT-PCR. The data are expressed as TNF-α copy number per 10 ng cDNA based on a standard curve using Roche LightCycler software.

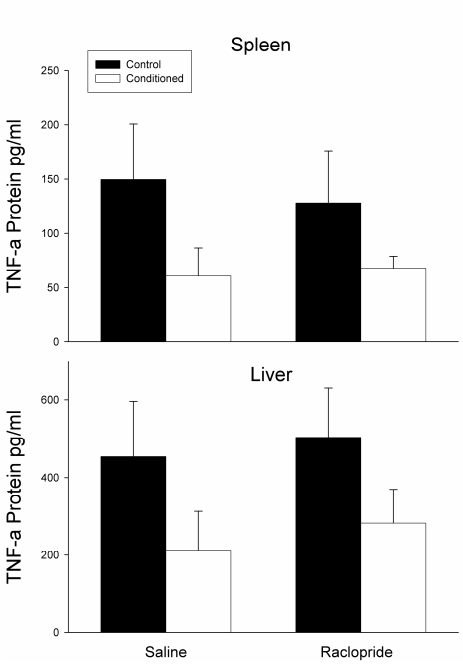

Figure 4.12 Effect of treatments on LPS-induced expression of TNF-α protein in the spleen and liver as determined by ELISA. The data are expressed as pg of protein per mL.

the saline-treated group exposed to the conditioned stimulus on test day exhibited significantly lower levels of TNF-α mRNA and protein in the spleen and TNF-α mRNA in the liver ($p<0.05$) when compared to the saline-treated control group. In addition, the raclopride treated group that was re-exposed to the conditioned stimulus on test day also exhibited a conditioned reduction in TNF-α mRNA and protein in the spleen and mRNA in the liver as compared to the saline-treated control group ($p<0.05$).

Discussion

The findings presented in the current chapter demonstrate that dopamine within the basolateral amygdala plays a critical role in the conditioned effects of heroin on proinflammatory mediators. Specifically, these results revealed that antagonism of dopamine D_1 receptors within the BLA blocks the expression of heroin-induced conditioned suppression of nitric oxide, TNF-α and IL-1β. The SCH23390 compound has been used extensively as a selective D_1 receptor antagonist and the dose of 2.0 μg/side was chosen based upon an established literature showing an effect of this dose administered intra-BLA on cue-induced reinstatement to drug seeking (Berglind et al., 2006; Alleweireldt et al., 2006). Interestingly, selective antagonism of dopamine D_2 receptors within this same region does not appear to have any effect as the animals exposed to the conditioned stimulus and administered the antagonist still exhibited a conditioned suppression in proinflammatory mediators. Collectively, these data indicate a role for dopamine D_1, but not D_2, receptors in the expression of heroin-induced conditioned immunomodulation. These data are in line with similar studies demonstrating that intra-accumbens SCH23390 blocks the effects of opioid drugs, and

stimuli associated with opioid drugs, on NK cell activity and nitric oxide production (Saurer et al., 2006; Saurer et al., 2009).

The mesolimbic dopamine system is known to be critically involved in the expression of the rewarding properties of drugs of abuse, including opiates. In fact, heroin administration indirectly induces increased dopamine by the binding of its metabolites to mu-opioid receptors on GABA-ergic neurons within the ventral tegmental area. Dopamine has also been widely implicated in the formation of memories underlying associative learning processes. Specifically, dopamine and its receptors have been shown to mediate the learning of associations between drugs of abuse and their conditioned cues. For example, microdialysis studies revealed an increase in dopamine and its metabolites during the learning of a discriminative task in rats (Hori et al., 1993) and upon the presentation of a stimulus that had previously been paired with cocaine administration (Weiss et al., 2000). In addition, intra-amygdalar 6-hydroxydopamine injections impaired (Ashford & Jones, 1976), while post-training intra-BLA dopamine infusions enhanced (LaLumiere et al., 2004) memory consolidation of a conditioned avoidance task. Furthermore, intra-BLA infusions of amphetamine were found to potentiate cue-induced drug-seeking behavior following extinction (Ledford et al., 2003). Taken together, these data suggest a role for dopamine in the BLA in heroin-induced classically conditioned immune alterations.

The study of classical or Pavlovian conditioning allows for the dissociation of the associative processes underlying the acquisition of a learned response from the mechanistic control of the expression of the response itself. In many cases these two phenomena are controlled via separate mechanisms. For example, antagonism of beta-adrenergic receptor activity disrupts the expression but not the acquisition of

conditioned morphine-induced immune alterations (Coussons-Read et al., 1994). The data reported here suggest that dopamine D_1 receptors within the BLA are required for the expression of heroin's conditioned effects on proinflammatory mediators. However, it is unknown whether D_1 receptors within the BLA might also be involved in the acquisition of these responses and this presents an important area for future investigation. Interestingly, there appears to be differential involvement of dopamine receptor subtypes within the BLA in the acquisition and the expression of responses to conditioned drug cues. Several subtypes of dopamine receptors have been characterized and the BLA has been shown to express both D_1- and D_2-like receptors (Meador-Woodruff et al, 1991; Scibilia et al, 1992). Macedo et al. (2007) demonstrated that antagonism of dopamine D_1 receptors within the BLA reduces the expression of conditioned, but not unconditioned, fear. In addition, stimulation of dopamine D_1 receptors in the BLA is necessary for the expression of cue-induced reinstatement of cocaine-seeking behavior in rats (e.g., See et al., 2001; Yun & Fields, 2003). In contrast to the role of D_1 receptors, D_2 receptors in the BLA appear to be involved in the acquisition of associations between drugs and the cues that guide subsequent cue-induced cocaine-seeking behavior (Berglind et al., 2006). There has also been evidence for the involvement of dopamine D_3 receptors within the BLA as antagonism of these receptors was found to block reinstatement of drug seeking under a second-order schedule of reinforcement (Di Ciano, 2008). Based on the evidence from these studies it might also be important to examine the potential role of dopamine D_2 and D_3 receptors in the acquisition of associations between heroin and heroin-related cues that elicit immunomodulation.

The BLA receives dopaminergic inputs from the ventral tegmental area (VTA) and substantia nigra as one of the main targets of the mesolimbic dopamine system

(Rosenkranz & Grace, 1999). These inputs appear to be important for learning to associate the rewarding properties of drugs of abuse with external cues resulting in the ability of these drug cues to induce relapse to drug-seeking and other conditioned behaviors (DiCiano & Everitt, 2005; See, 2005). The data reported here suggest that opioids and opioid-associated drug cues induce dopamine release from neurons originating in the VTA or substantia nigra which then binds to D_1 receptors located within the BLA to influence the expression of conditioned responses to heroin related cues. Research has shown that the primary targets of mesolimbic dopaminergic neuron inputs to the BLA are the dendrites of the pyramidal neurons (Muller et al., 2009). Within the BLA there exist two distinct neuronal cell populations, pyramidal and non-pyramidal neurons. The pyramidal neurons of the BLA primarily utilize glutamate as their neurotransmitter and exhibit spiny projections while the non-pyramidal cells rely heavily upon inhibitory GABA signals and show fewer spines (McDonald, 1992). Dopamine selectively increases the excitability of BLA pyramidal neurons allowing for potentiation of the inputs from other brain regions, such as the cortex (Pickel et al., 2006). Interestingly, the pyramidal cell glutamatergic projections from the BLA have been shown to synapse in close proximity to dopamine axons on medium spiny neurons of the nucleus accumbens (Johnson et al., 1994; Kelley et al., 1982; Robinson & Beart, 1988) thus giving rise to a complex interaction between dopamine and glutamate within these two brain regions. In addition, electrophysiological studies have shown that tetanic stimulation of the BLA evokes dopamine efflux in the nucleus accumbens via glutamate receptor-dependent mechanisms localized within the accumbens (Floresco et al., 1998). Furthermore, either exogenously applied or synaptically released dopamine can modulate excitatory responses of nucleus accumbens neurons evoked by low frequency stimulation of the BLA (Yim &

Mogenson, 1982; 1986). Collectively, these data suggest that the circuit encompassing the VTA, BLA and nucleus accumbens may be involved in the conditioned effects of heroin on immune measures.

While it appears that the BLA does not directly impact immune functioning it is highly plausible that conditioned immune alterations may be induced through the connections of the BLA with the nucleus accumbens. Saurer et al. (2006a) demonstrated that intra-accumbens shell administration of a dopamine D_1 agonist resulted in decreased NK cell activity identifying this brain area as having the ability to directly induce immunomodulation. It is as yet unknown what factors may be mediating these effects in the periphery but there is a wealth of data suggesting that the sympathetic nervous system may be involved. For example, both primary and secondary lymphoid tissues have been found to be innervated by the sympathetic nerve fibers (Williams et al., 1981; Felten et al., 1984) and adrenergic receptors have been identified on the surface of immune cells (Livnat et al., 1985; Hori et al., 1997; Maestroni, 2006). More specifically, peripheral administration of β-adrenergic antagonists has been shown to block the expression of the conditioned effects of morphine on several measurements of immune status (Coussons-Read et al., 1994). However, it appears that at least some of these immune alterations are mediated peripherally by neuropeptide Y as systemic administration of a neuropeptide Y Y_1 receptor antagonist dose-dependently attenuates morphine-induced alterations in NK cell activity while β-adrenergic antagonism had no effect (Saurer et al., 2006b). These data seem to suggest differential mechanisms for the control of specific opioid-induced immune alterations.

It is important to understand the mechanisms by which heroin-induced conditioned immunomodulation occurs as these effects may have widespread

implications for the health of recovering addicts. Nitric oxide, TNF-α and IL-1β are all components of the innate immune response and play critical roles in the host's initial response to pathogenic challenge. In the absence of a proper immune response, which may occur following exposure to drug-related cues, the host's immune system may not be able to adequately deal with infection which may allow for increased pathogenic proliferation and sepsis. The exact neural mechanism through which heroin-induced conditioned immunomodulation occurs is still not fully understood, but the data presented here suggests that the BLA plays a critical role. The experiments reported here are the first to identify the dopaminergic D_1 receptors within the BLA as crucial for the expression of the effects of heroin-related cues on immune functioning.

CHAPTER 5

GENERAL DISCUSSION

Experimental Findings

Collectively, the present studies provide important new data regarding the neurobiological mechanisms by which heroin conditioned immunomodulation occurs. The investigation of conditioned responses to drug cues has led to significant advancements in our understanding and treatment of opiate addiction. . However, the conditioned immunomodulation that may occur upon exposure to these same drug cues and the implications that these changes in immune functioning have on susceptiblity to disease is a relatively neglected area of investigation. The results of these studies suggest that exposure to heroin-paired stimuli can have a profound influence on the proinflammatory mediators of the innate immune system. The implication is that former and current opiate users may be more susceptible to infection not only because of the direct action of opiates on target tissues but also because their body associates non-drug cues with changes in immunity. The expression of these conditioned responses seem to be mediated by the same neural substrates found to be in control of other conditioned responses to drugs of abuse.

Previously published data (Lysle & Ijames, 2002) and the results presented in Chapter 2 show that the suppressive effects of heroin on proinflammatory mediators can be conditioned to environmental stimuli and confirms this conditioned response is an example of associative learning. This conclusion is suppported by studies showing conditioned suppression in nitric oxide production is susceptible to the effects of both

extinction and latent inhibition. Extinction is the reduction in a conditioned response observed following repeated exposure to the conditioned stimulus without the unconditioned stimulus. Thus, extinction occurs when the conditioned stimulus no longer reliably predicts drug availability, and as result, the conditioned response is reduced. Latent inhibition is the result of repeated exposure to the conditioned stimulus prior to the association of the conditioned stimulus with a drug; in latent inhibition, a subject must overcome the learned irrelevance of the stimlus in order to associate the stimulus with a physiological response. The experiments in Chapter 2 established that the conditioned responses observed upon exposure to a previously heroin-paired environment are a true example of classical conditioning by providing evidence of extinction and latent inhibition. The results indicate that these conditioned responses involve associative learning processes which led to the investigation of the involvement of brain areas and neurotransmitter systems known play a role in learned associations between drugs of abuse and their cues.

Given the results of Chapter 2, Chapter 3 sought to explore the potential involvement of the basolateral amygdala (BLA) in the expression of the conditioned effects of heroin on nitric oxide. The BLA is a critical component of the neural circuitry mediating conditioned responses to drugs of abuse. Animals receiving morphine injections paired with a distinct environment showed greater Fos activation within the BLA when re-exposed to that environment than animals receiving un-paired injections (Harris & Ashton-Jones, 2003). Similarly, cocaine-associated cues elicited neural activity within the BLA nearly identical to that seen upon intravenous delivery of cocaine in rats taught to self-administer the drug (Carelli et al., 2003). Together, these and similar studies provide clear and consistent rationale supporting the investigation of the BLA as part of the neurobiological circuitry involved in the conditioned immune

responses to drugs of abuse, in general, and heroin, in particular. In addition, the experiments in Chapter 3 demonstrate that the effects of heroin on peripheral TNF-α and IL-1β expression may be conditioned to enviroinmental stimuli. Given the multifaceted and complex involvement of these cytokines, as well as nitric oxide, in the initial immune response to infectious challenge it is essential to understand the mechanisms and neural circuitry through which heroin's conditioned alterations of these pro-inflammatory mediators are controlled. The results from the experiments in Chapter 3 indicate that temporary inhibition of the BLA attenuates the conditioned effects of heroin on the proinflammatory mediators, nitric oxide, TNF-α and IL-1β, thereby suggesting that the BLA may mediate these examples of neuroimmunomodulation in conditioned animals.

Chapter 4 demonstrates the role of dopamine receptors within the BLA for the conditioned effects of heroin on nitric oxide and the proinflammatory cytokines, TNF-α and IL-1β. Specifically, intra-BLA adminstration of the D_1 receptor antagonist, SCH23390, reduced the conditioned response in animals exposed to a previously heroin-paired environment. Conversely, intra-BLA administration of the D_2 receptor antagonist, raclopride, had no effect on the conditioned response as animals receiving the drug and re-exposed to the conditioning chamber still exhibited significant conditioned reductions in nitric oxide, TNF-α and IL-1β. These results are consistent with Saurer et al. (2008) in which conditioned morphine-induced alterations in natural killer (NK) cell activity were found to be mediated by dopamine D_1 receptors in another brain region (the nucleus accumbens shell). Interestingly, D1 receptors mediate the non-conditioned alterations in NK cell cytotoxity observed following morphine administration (Saurer et al., 2006). Collectively, these data identify D_1

receptors within the BLA as part of the circuitry that must be activated for the expression of heroin-induced conditioned immunomodulation.

Neural Circuitry

The experiments outlined in this dissertation indicate a clear role for the BLA in mediating the expression of conditioned immune alterations observed upon exposure to a previously heroin-paired environment. These data show that activation of dopamine D_1 receptors is necessary for the expression heroin-induced conditioned immunomodulation. The circuitry mediating the conditioned effects of heroin related stimuli on immune paramaters may be similar to the circuitry mediating other conditioned responses to drugs of abuse. Studies have shown that stimuli associated with drug use may acquire incentive-motivational properties and may elicit drug craving and/or seeking (Weiss, 2005; Sell et al., 2000). Animal models such as the conditioned place preference test are used to observe these drug associated behaviors in rodents and to investigate the neural mechanisms by which these behaviors are controlled. Through the use of these models several brain regions have been identified that may exist as part of a circuit mediating cue-induced drug seeking behavior; these regions include the prelimbic region of the prefrontal cortex, anterior cingulate cortex, the central and basolateral amygdala, the ventral tegmental area (VTA), hippocampus, hypothalamus and the nucleus accumbens (Zijlstra et al., 2009; Chaudhri et al., 2008; Hamlin et al., 2008; Kalivas & Volkow, 2005).

The VTA is especially important for mediating the rewarding properties of conditioned stimuli that have become associated with drugs of abuse. For example, dopaminergic neurons in the VTA increase firing rates in response to reward-related conditioned stimuli (Kosobud et al., 1994) and increases in mesolimbic dopamine are

observed prior to the reinstatement of responding for reward (Gratton and Wise, 1994). The VTA is the primary source of mesolimbic dopamine which is found to be increased in VTA target regions, such as the nucleus accumbens and amygdala, following exposure to conditioned cues predicting drug availability (Ranaldi et al., 1999; Harmer & Phillips, 1999; Ito et al., 2000). Potentiation of VTA dopaminergic neurons and increases in VTA dopaminergic transmission are attributed to intra-VTA glutamatergic activity as glutamate antagonists administered directly into the VTA, but not outside of it, inhibit the acquisition of conditioned place preference to drugs of abuse (Harris & Aston-Jones, 2003; Shabat-Simon et al., 2008). In addition, it is possible that alterations in dopamine activity are controlled by the presence of glutamate receptors on the VTA dopaminergic neurons as glutamate receptors have been implicated in the control of persistent cocaine seeking behaviors (Engblom et al., 2008). However, Engblom et al. (2008) focused on unconditioned cocaine seeking behavior and it is not clear whether conditioned drug seeking behavior would utilize this same mechanism. A potential caveat is that the activation of glutamate receptors on VTA dopaminergic neurons may be specific to the pharmacodynamic properties of cocaine and may not be generalizable to other drugs of abuse. Nevertheless, the dopaminergic connections between the VTA and its targets are important for mediating conditioned responses to all drugs of abuse.

The research presented here as well as by others elucidates a role for the BLA in the conditioned responses to drugs of abuse. For example, exposure to drug cues will reinstate drug seeking behavior in rats even after extinction training (Gracy et al., 2000) and inactivation of the BLA will block the ability of these cues to reinstate drug seeking (McLauglin & See 2003; Fuchs et al 2005). Interestingly, there was no effect of BLA inactivation on self-administration suggesting that the BLA is critical for

conditioned but not unconditioned reinforcement (Meil & See, 1997). It is likely that while the connections between the VTA and the nucleus accumbens modulate the unconditioned effects of drug administration, the circuits connecting the VTA to the BLA and the BLA to the nucleus accumbens may mediate the conditioned responses to drug cues. In fact, the BLA is one of the main targets of the mesolimbic dopaminergic system and receives numerous projections from the VTA (Brinley-Reed & McDonald, 1999). The BLA also receives input from the prefrontal cortex and stimulation of the medial prefrontal cortex results in activation of BLA GABAergic neurons and a reduction in BLA neuronal excitability which is reversed by the presence of dopamine (Grace & Rosenkranz, 2002). Thus, these two BLA circuits appear to differentially modulate the plasticity of the BLA and its outputs. In addition, glutamatergic projections from the BLA synapse in close proximity to dopaminergic neurons of the nucleus accumbens (Johnson et al., 1994).

The nucleus accumbens, one of the other main targets of VTA dopaminergic neurons, is another brain structure that has been widely established as critical for conditioned responses to stimuli associated with drug abuse. The nucleus accumbens receives dopaminergic inputs from cells originating in the VTA as well as the glutamatergic projections from the BLA. The neural circuitry from the VTA to the BLA and the BLA to the nucleus accumbens appears to especially important for the acquisition and expression of conditioned responses to addictive drugs. In support of this, pharmacologically disconnecting the BLA from the nucleus accumbens reduces cue-induced drug seeking (Di Ciano & Everitt, 2004). This was accomplished by antagonizing the dopamine receptors within the BLA to block the influence of VTA dopaminergic input on one side of the brain while simultaneously antagonizing the glutamatergic input from the BLA to the nucleus accumbens on the contralateral side.

Interestingly, the nucleus accumbens may be part of the circuitry mediating opiate-induced conditioned immune alterations (Saurer et al., 2008). In addition, the accumbens appears to be involved in the regulation of peripheral immune measures as lesions or pharmacological manipulations of mesoaccumbens dopaminergic neurons result in altered immune reactivity (Deleplanque et al., 1994; Nistico et al., 1994). Furthermore, both conditioned and unconditioned effects of morphine appear to be mediated via dopamine D_1 receptors in the nucleus accumbens as antagonism of these receptors block morphine- and morphine-associated stimuli induced suppression of NK cell activity (Saurer et al., 2006a; Saurer et al., 2008). An investigation of the contribution of the VTA-BLA-accumbens circuit in conditioned immune alterations seen upon exposure to heroin-related stimuli will be an important next step in modeling the neurobiology of addiction and addiction-related changes in physiology.

Peripheral Mediators

While the exact peripheral mechanisms modulating the alterations in proinflammatory mediators observed in response to exposure to opioid-associated drug cues are unknown, research has revealed potential roles for several neuroendocrine factors. The sympathetic nervous system innervates both primary and secondary lymphoid tissues and releases molecules that interact with receptors on immune cells (Williams et al., 1981; Felten et al., 1984). In fact, sympathetic innervation of the spleen, provides some of the most concrete evidence for communication between the nervous and immune systems through sympathetic nervous system release of norepinephrine (and other chemicals) from nerve fibers onto immune cells in this organ. In addition, several researchers have indicated a role for the sympatehtic nervous system for the expression of conditioned immune

alterations. For example, the immunosuppressive effects of cyclosporine A can be conditioned to a gustatory stimulus and this conditioned effect is blocked by denervation of the spleen (Exton et al., 1998). Previous research from our laboratory shows that the suppression of splenic mitogenic responsiveness observed following exposure to a conditioned aversive stimulus can be blocked by administration of the beta-adrenergic receptor antagonist, propanolol (Lysle et al., 1991). Furthermore, the sympathetic nervous system may also be involved in the direct effects of morphine on immunity as both selective and non-selective beta-adrenergic receptor antagonists attenuate the suppressive effects of morphine administration on select measures of lymphocyte proliferation (Fecho et al., 1993). Other experimentation reveals that both the conditioned and unconditioned effects of opiates on the cytotoxicity of NK cells appears to be mediated by neuropeptide Y, a peptide transmitter co-released with norepinephrine by the sympathetic nervous system (Saurer et al. 2006; 2008). In addition, the expression of a subset of morphine-induced conditioned immune alterations are blocked by the systemic administration of the non-selective peripheral β-adrenergic receptor antagonist, nadolol (Coussons-Read et al., 1994). Collectively these data suggest a role for the sympathetic nervous system in the modulation of opiate-induced conditioned immunomodulation.

Hypothalamic-pituitary-adrenal (HPA) axis activiation may result in alterations in immune functioning through the release of hormones from the hypothalamus, pituitary and adrenal gland. Stress is a potent activator of the HPA axis and is immunomodulatory in part through stress-mediated release of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids (from the hypothalamus, pituitary, and adrenal, respecitvely). For instance, psychological stress in animals induces decreases in the immune cytokine

interferon-gamma and these decreases are reversed when the animals are treated with a glucocorticoid receptor antagonist (Curtin et al., 2009) HPA axis factors are also involved in with some instances of conditioned immune alterations. For example, exposure to a stimulus previously paired with LPS administration induces suppression of IL-2 production concomitantly with increases in circulating corticosterone (Janz et al., 1996) and exposure to a stimulus previously associated with an electic shock treatment elicits reductions in NK cell activity as well as increases in plasma corticosterone. However, HPA axis activation may not be necessary for the expression of all conditioned immunological effects (Perez & Lysle, 1995) as the effects on NK cell activity are blocked by adminstration of a CRH antagonist while the effects on plasma corticosterone are not. Instead, CRH may be acting through alternative immunomodulatory pathways. One possibility is the locus coeruleus which is activated by CRH and CRH adminstration into this brain region results in decreases in T-lymphocyte mitogenic responses that are associated with decreases in antibody production (Rassnick et al., 1994). In regard to conditioned immunomodulation, cocaine-associated cues induce alterations in immune functioning, including TNF-α production, and these alterations accompany elevations in corticosterone levels suggesting the HPA axis may be involved in drug cue mediated conditioned immunomodulation (Kubera et al., 2008).

A third possible drug cue mediated conditioned immunomodulation is the endogenous opioid system. As mentioned previously, exposure to a conditioned stimulus previously paired with electrical shock results in the suppression of a number of immune parameters including NK cell activity, mitogen-induced lymphocyte proliferation and the number of antibody producing cells (Lysle et al., 1988; Lysle et al., 1990). These effects appear to be mediated by opioid receptors in the brain as

systemic administration of the opioid antagonist, naltrexone, but not *N*-methylnaltrexone[5] results in an attenuation (Lysle et al., 1992). More specifically, reductions in immunity are mediated by mu-opioid receptors as intracerebroventricular administration of a mu-1 selective opioid receptor antagonist, but not a kappa- or delta- selective antagonist, blocks electrical shock-induced conditioned alterations in immunity (Perez & Lysle, 1997). Both the acquisition and the expression of morphine-induced conditioned suppression of immune functioning are attenuated by antagonism of opioid receptors, suggesting endogenous opioids play a role in opioid-induced conditioned immunomodulation (Coussons-Read et al., 1994). While the mechanisms underlying the conditioned effects of opiate drugs on immunity remain unknown, there are several possibilities as indicated by the wealth of data surrounding the mediators of conditioned responses to non-drug related stimuli. The continued search for the exact peripheral mediators modulating the conditioned effects of opioid drugs on immune paramaters is critical for understanding how to control these effects in recovering addicts and reduce the susceptibility to infection in these individuals.

General Conclusions

There is a substantial literature indicating that heroin use has a profound, negative impact on clinical health outcomes. Given the widespread use of illicit heroin, as well as the clinical use of other opioids, it is important to understand not only the direct effects of these chemicals on target tissues but also the mechanisms underlying the conditioned effects of these drugs on physiological processes,

[5] N-methylnaltrexone is a quaternary form of naltrexone that does not readily cross the blood-brain barrier

including immune functioning. The experiments in this dissertation are among the first to show that exposure to stimuli previously associated with heroin use may not only contribute to relapse but may also alter the ability of the host's immune system to respond to an infectious agent. In addition, the data provide new and revealing information regarding the neural circuitry underlying Pavlovian conditioning processes, specifically those in which the unconditioned stimulus is a potentially addictive drug. Furthermore, as the capacity to learn associations between drug administration and environmental stimuli is shared by virtually all drug related conditioned responses, the results from these studies suggest mechanistic pathways for how stimuli associated with drugs such as cocaine, amphetamine, and alcohol may change on immune responses, i.e., by acting within or through systems associated with the basolateral amygdala.

Much of the current research examining the mechanism of drug addiction and treatment focuses on reducing relapse by attenating cue induced drug seeking behavior. However, it is imperative to consider the immunomodulatory effects drug cues can have on immune functioning. The results presented here provide the first evidence for a neurological circuit mediating the immunologic alterations associated with drug cues paired with heroin administration. Drugs users are not only influenced by the action of drugs on target cells, but drug users are also influenced by unique (to each user) drug-associated cues. As drug-associated cues can be immunomodulatory and are often immunosupressive. This suggests drug users (and recovering addicts) may experience alterations in their ability to combat infectious diseases even in the absence of drugs. The user-specific nature of these results suggests the host response to opioids is not be static, but will change as the organism learns to anticipate drug adminstration. It follows that recovering drug addicts may be at an increased risk for

developing opportunistic infections as well as for other problems from decreased immunity such as improper antibody responses to vaccination. These effects need to be factored into the comprehensive treatment of both current and former opiate users.

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